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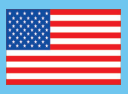

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EDITORIAL

Post-traumatic stress disorder risk and brain-derived neurotrophic factor Val66Met

Lei Zhang, Xiao-Xia Li, Xian-Zhang Hu

Lei Zhang, Xiao-Xia Li, Xian-Zhang Hu, Center for the Study of Traumatic Stress, Department of Psychiatry, Uniformed Services University of the Health Sciences, Bethesda, MD 20814, United States

Author contributions: All authors equally contributed to this paper with conception, literature review and analysis, drafting and critical revision and editing, and final approval of the final version.

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Telephone: +1-301-2950921
Fax: +1-301-2950923

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Abstract

Brain-derived neurotrophic factor (BDNF), which regulates neuronal survival, growth differentiation, and synapse formation, is known to be associated with depression and post-traumatic stress disorder (PTSD). However, the molecular mechanism for those mental disorders remains unknown. Studies have shown that BDNF is associated with PTSD risk and exaggerated startle reaction (a major arousal manifestation of PTSD) in United States military service members who were deployed during the wars in Iraq and Afghanistan. The frequency of the Met/Met in BDNF gene was greater among those with PTSD than those without PTSD. Among individuals who experienced fewer lifetime stressful events, the Met carriers have significantly higher total and startle scores on the PTSD Checklist than the Val/Val carriers. In addition, subjects with PTSD showed higher levels of BDNF in their peripheral blood plasma than the non-probable-PTSD controls. Increased BDNF levels and startle response were observed in both blood plasma and brain hippocampus by inescapable tail shock in rats. In this paper, we reviewed these data to discuss BDNF as a potential biomarker for PTSD risk and its possible roles in the onset of PTSD.

Key words: Post-traumatic stress disorder; Brain-derived neurotrophic factor; Depression; Biomarker; Startle

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Core tip: Brain-derived neurotrophic factor (BDNF), which regulates neuronal survival, growth differentiation, and synapse formation, is known to be associated with depression and post-traumatic stress disorder (PTSD). However, the molecular mechanism for those mental disorders remains unknown. In this paper, we reviewed these data to discuss BDNF as a potential biomarker for PTSD risk and its possible roles in the onset of PTSD.

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INTRODUCTION

Brain-derived neurotrophic factor (BDNF), first discovered in the early 1980s, is considered a member of the nerve growth factor family of neurotrophins[1], which have important roles in the development, physiology, and pathology of mental disorders[2,3]. BDNF is expressed in a number of tissues and cell types, including the brain and blood[4]. In recent years, BDNF has been implicated in a number of psychiatric disorders, such as depression, anxiety, eating disorders, and posttraumatic stress disorder (PTSD) (Table 1). In this review, we will focus on the findings of the association between BDNF and PTSD and expand upon our recent works to provide an argument for the potential role of BDNF in additional psychiatric disorders with their roots in emotional dysregulation, specifically PTSD.

BDNF is a precursor protein (proBDNF) that is proteolytically cleaved to generate mature BDNF[5] *via* tissue-type plasminogen activator (tPA)/plasminogen[6]. P11 (S100A10), a component of the Annexin Ⅱ and PTSD associated gene[7,8], greatly enhances the activation of plasmin by tPA[6]. It is suggested that p11 may act through the tPA/plasminogen/BDNF pathway to achieve its antidepressant effect[6]. BDNF binds to either of two functionally different classes of cell surface receptors, the TrkB receptor tyrosine kinase or the p75 neurotrophin receptor (p75NTR), a member of the tumor necrosis factor receptor super family[9]. ProBDNF and mature BDNF differentially interact with the TrkB receptor tyrosine kinase or the p75NTR, respectively[9,10]. ProBDNF induces neuronal apoptosis *via* activation of a receptor complex of p75NTR and sortilin[11]. ProBDNF induced p75NTR signaling gives rise to an increase in c-Jun N-terminal kinase and nuclear factor κB, regulating apoptosis, axonal retraction, or the pruning of dendritic spines[12]. BDNF-induced TrkB receptor signaling regulates neurotrophic responses *via* rapid activation of the PI-3 kinase, Ras/MAPK, and Phospholipase C-γ pathways; therefore influencing transcriptional events that affect the cell-cycle, neurite outgrowth, and synaptic plasticity (Figure 1), suggesting that the BDNF plays a key role in stress response and stress-related behaviors[13].

BDNF AND PTSD

In the animal model, it is found that BDNF protein was over-expressed in the plasma and hippocampus of stressed rats compared with non-stressed controls. These data are in agreement with others showing that stress results in BDNF over-expression in the hippocampus, leading to the hypotheses of a BDNF-related compensatory mechanism[14] and the blood levels of BDNF mirror the hippocampus levels induced by traumatic stress[15]. The rats with up-regulated BDNF in both blood and hippocampus induced by inescapable tail shock demonstrated increases of startle response[15]. Acoustic challenge is known to trigger a range of physiological responses, including startle. The startle reaction (also known as the startle response, the startle reflex, or the alarm reaction) is the psychological and physiological response to a sudden unexpected stimulus, such as a flash of light, a loud noise (acoustic startle reflex), or a quick movement near the face. Abnormality of the startle response, which results from an elevated activation of the autonomic nervous system, is a core symptom of PTSD-hyperarousal[16,17]. These data from the studies in animal model suggests that both BDNF and stress play important roles in startle response, although the mechanism needs to be further analyzed.

Within the last several years, the biological basis of PTSD has been an important focus of research in psychiatry due to the Iraq and Afghanistan wars. There are data showing that the blood BDNF is a potential biomarker for PTSD, the traumatic stress-related disorders, and debilitating psychiatric disorders[4,15,18-21]. A common single nucleotide polymorphism (SNP) in the BDNF gene leading a valine to methionine substitution at position 66 (Val66Met) influences human hippocampal volume[22], memory[23] and susceptibility to PTSD[23]. The BDNF Val66Met polymorphism is associated with sense of coherence, a presumed stress-related protective cognition in a non-clinical community sample[24]. Individuals carrying the Met had decreased activity-dependent BDNF secretion from neurons, leading to impairment of learning[22]. Recently, a study demonstrated that the frequency distribution of Val66Met polymorphism was different between subjects with and without PTSD[15]. The frequencies of Met/Met genotype and Met carriers are significantly higher in individuals with PTSD than those without PTSD. The allelic frequency of Met was two-fold higher (33.3% *vs* 17.5%) in individuals with PTSD than in non-PTSD controls, supporting the notion that Met carriers have a smaller hippocampal volume relative to Val/Val homozygous[25-27] and decreased volumes in the temporal and occipital lobe grey matter[28].

These data suggest a role of BDNF in the plasticity of the brain, which might be associated with PTSD. It was found that Met carriers performed more poorly than control subjects (Val/Val carrier) on the memory tasks[29]. The interaction of Met-allele and stress can result in depression, anxiety and arousal[30]. There is a significant three-way interaction between Val66Met, serotonin transporter linked promoter region (5-HTTLPR) and maltreatment history in predicting depression[31]. Children with the Met allele and two short alleles of 5-HTTLPR demonstrated the highest depression scores. However, the vulnerability associated with these two genotypes was only evident in the maltreated children[31]. There is a report showing that veterans with psychotic PTSD carried more Met alleles of the BDNF

Table 1 Brain derived neurotrophic factor and common psychiatric disorders

Mental disorders	Results and references
Schizophrenia	Polymorphisms and BDNF[39]
	The Val66Met allele association[40]
	The TrkB receptor decreased in the hippocampus[41]
	Up-[41] and down[42] regulated BDNF in the frontal cortices
Major depressive disorder	Antidepressant increases BDNF levels[43]
	BDNF protein increased in the NAc[44]
	The decreased prefrontal cortex is correlated with decreased BDNF and TrkB levels[45,46]
	BDNF dose-dependently decreases 5HT uptake[47]
	Unclear whether BDNF polymorphisms contribute to expression of MDD symptoms or antidepressant efficacy[48,49]
Bipolar	Both lithium and valproic acid increase BDNF expression in corticolimbic brain[40]
	BDNF protein levels decreased in post mortem hippocampal tissue[40]
	Serum BDNF levels decreased[49]
	The V66M BDNF allele strongly correlated to BD[35,50]
PTSD	PTSD risk associated with BDNF Val66Met and BDNF overexpression[15]
	Blood BDNF levels and PTSD[4,15,18-21]

NAc: Nucleus accumbens; BDNF: Brain derived neurotrophic factor; MDD: Microgram per square decimeter per day; PTSD: Post-traumatic stress disorder.

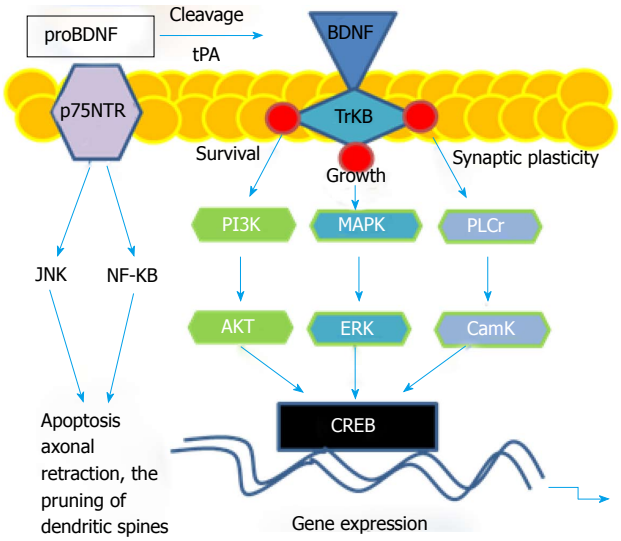

Figure 1 Brain-derived neurotrophic factor signaling pathway. The binding of proBDNF to the p75NTR up-regulates c-Jun N-terminal kinase and nuclear factor κB, triggering apoptosis, axonal retraction, or the pruning of dendritic spines. The proBDNF is cleaved to generate mature BDNF *via* tPA/plasminogen. When BDNF binding to its receptors, the receptor tyrosine kinase TrkB becomes phosphorylated, leading to phosphorylation at various sites or activation of downstream pathways. Such activation shows at PI3K sites, which activates AKT, regulating cell survival. The activation of MAPK/ERK leads to cell growth and differentiation. The activation of PLC-γ pathway regulates IP3 receptor to release intracellular calcium stores, in turn to enhance CamK activity, regulating synaptic plasticity. All three pathways converge on transcription factor CREB, binging to certain DNA sequences called CRE, playing a key role in BDNF-induced gene expression. BDNF: Brain derived neurotrophic factor; PTSD: Post-traumatic stress disorder; tPA: Tissue-type plasminogen activator; CamK: Calmodulin kinase; CAMP: Cyclic adenosine monophosphate; CREB: CAMP response elements; CRE: CAMP response elements; AKT: Protein kinase B; IP3: Inositol trisphosphate.

Val66Met than non-psychotic veterans with PTSD or veterans without PTSD[32]. This further supports the linkage between BDNF and PTSD, especially in a military population at war[15]. However, not all studies show the same results. There is a case-control genetic association study showing no relationship between BDNF Val66Met and PTSD diagnosis[23]. It is possible the difference in frequency of trauma exposure, age, and other study conditions among the participants in the studies can explain these divergent results[33]; however, this remains to be determined.

In an association study between BDNF Val66Met and the startle score of PTSD Checklist (PCL), a core symptom of hyperarousal in PTSD is observed to be associated with the polymorphism. The distribution of the Met/Met frequency was significantly different between those with and without exaggerated startle[15]. The frequency of the Met/Met genotype was almost four-fold (12.2% *vs* 3.3%) higher in subjects with exaggerated startle than in those without exaggerated startle. In addition, the frequency of the Met allele was higher in subjects with exaggerated startle than in those without exaggerated startle (24.4% *vs* 15.3%), indicating that Met/Met is associated with hyperarousal vulnerability[15]. Since the frequency of Val/Val genotype is higher in the non-startle group than in those endorsing startle reactions, it suggests that Val/Val is related to protection from exaggerated startle reactions and perhaps PTSD. Subjects with fewer stressful life events and carrying the Met/Met homozygote have significantly higher startle scores than those Val carries, indicating that less exposure to stressful life events is associated with higher risk of hyperarousal in Met/Met carriers, but lower risk of hyperarousal in Val carriers[15].

It is found that the BDNF Val66Met does not significantly effect on the PCL total score in the subjects who experienced higher (four or more) stressful life events. However, among those subjects who reported fewer exposures of stressful life events, the Met carriers show higher PCL total scores (*i.e.*, reported greater PTSD symptoms) than Val carriers. Therefore, at a lower exposure of stressful life events, Met carriers had a higher risk of PTSD symptoms, and the presence of Val led to a lower risk of PTSD symptoms[15]. This indicates that there are protective effects at higher levels of

stress exposure. A similar phenomenon is observed in a catechol-O-methyltransferase gene association study, which showed that those homozygous for the Met allele demonstrated a high risk for PTSD, independent of the severity of traumatic load[34]. Alternatively, different neuronal mechanisms[35] may be active in minimally exposed and highly exposed individuals who develop PTSD, supporting different underlying trajectories of this disorder and perhaps different treatments[15].

It is also found that at protein levels, subjects with PTSD had significantly higher serum levels of BDNF than the non-PTSD controls[20]. In addition, the BDNF levels in Met carriers are higher than in Val/Val homozygotes[36]. The findings are consistent with other results showing that serum BDNF levels in individuals with PTSD are higher than in age and sex matched controls right after traumatic events[19,37]. However, some studies have shown either significantly lower levels of BDNF among those with PTSD[4] or significant difference of BDNF levels in serum between PTSD and non-PTSD controls[21]. These contradictory results may be due to the different methods used in the various studies. The samples may have been collected from dissimilar population[18,20,21,37], at different time points during the course of the disease[18,20,21,37], or from different animal models[38]. These assumptions need to be further analyzed. Nevertheless, these data suggest that BDNF is associated with PTSD risk at both translational and genomic levels[15]. Therefore, blood levels of BDNF may be of benefit in developing non-invasive diagnostics for PTSD[15].

CONCLUSION

The association between BDNF and PTSD has been suggested. The frequency of the Met/Met was greater among those with PTSD than non-PTSD controls. In addition, this SNP is associated with exaggerated startle, but not with other items on the PCL. Among individuals who experienced fewer lifetime stressful events, Met carriers have significantly higher total and startle scores on the PCL than Val/Val carries. At protein levels, subjects with PTSD had higher levels of BDNF in their peripheral blood plasma than the non-PTSD controls. In a rodent model, complementing the data from the human subjects, increased BDNF protein levels accompanied by an obvious elevation of the startle response were obtained in both blood plasma and brain hippocampus by inescapable tail shock. Therefore, protein BNDF in the blood and startle test, aside from genotype, and neuroimaging could also serve as biomarkers to direct more personalized PTSD treatment. Future studies on patient cohorts will elucidate whether these biomarkers, particular BDNF for PTSD prove to be useful in a clinical setting.

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EDITORIAL

Gender differences in caregiving among family - caregivers of people with mental illnesses

Nidhi Sharma, Subho Chakrabarti, Sandeep Grover

Nidhi Sharma, Subho Chakrabarti, Sandeep Grover, Department of Psychiatry, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India

Author contributions: Sharma N, Chakrabarti S and Grover S equally contributed to this paper.

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Correspondence to: Subho Chakrabarti, MD, MAMS, FRCPsych, Professor, Department of Psychiatry, Postgraduate Institute of Medical Education and Research, PGIMER, Sector-12, Chandigarh 160012, India. subhochd@yahoo.com
Telephone: +91-172-2756808
Fax: +91-172-2744401

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Abstract

All over the world women are the predominant providers of informal care for family members with chronic medical conditions or disabilities, including the elderly and adults with mental illnesses. It has been suggested that there are several societal and cultural demands on women to adopt the role of a family-caregiver. Stress-coping theories propose that women are more likely to be exposed to caregiving stressors, and are likely to perceive, report and cope with these stressors differently from men. Many studies, which have examined gender differences among family-caregivers of people with mental illnesses, have concluded that women spend more time in providing care and carry out personal-care tasks more often than men. These studies have also found that women experience greater mental and physical strain, greater caregiver-burden, and higher levels of psychological distress while providing care. However, almost an equal number of studies have not found any differences between men and women on these aspects. This has led to the view that though there may be certain differences between male and female caregivers, most of these are small in magnitude and of doubtful clinical significance. Accordingly, caregiver-gender is thought to explain only a minor proportion of the variance in negative caregiving outcomes. A similar inconsistency characterizes the explanations provided for gender differences in caregiving such as role expectations, differences in stress, coping and social support, and response biases in reporting distress. Apart from the equivocal and inconsistent evidence, there are other problems in the literature on gender differences in caregiving. Most of the evidence has been derived from studies on caregivers of elderly people who either suffer from dementia or other physical conditions. Similar research on other mental illnesses such as schizophrenia or mood disorders is relatively scarce. With changing demographics and social norms men are increasingly assuming roles as caregivers. However, the experience of men while providing care has not been explored adequately. The impact of gender on caregiving outcomes may be mediated by several other variables including patient-related factors, socio-demographic variables, and effects of kinship status, culture and ethnicity, but these have seldom been considered in the research on gender differences. Finally, it is apparent that methodological variations in samples, designs and

assessments between studies contribute a great deal to the observed gender differences. This review highlights all these issues and concludes that there is much need for further research in this area if the true nature of gender differences in family-caregiving of mental illnesses is to be discerned.

Key words: Gender; Family-caregiving; Schizophrenia; Elderly; Dementia; Mood disorders

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Core tip: Women form the bulk of those who provide care for people with mental illnesses. Many studies have found that they are more exposed to caregiving stressors and report greater strain, burden and distress than men. However, the evidence for such gender differences in caregiving is equivocal and inconsistent leading to the view that caregiver-gender explains only a minor proportion of the variance in negative caregiving outcomes. Moreover, the evidence is not representative and often methodologically flawed. There is, thus, much scope for further research to understand the true nature of gender differences in family-caregiving of mental illnesses.

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INTRODUCTION

Caring for someone with a mental illness has always been a family endeavour. This is true for developed as well as developing countries. Despite their relatively greater mental health-care resources, changing demographics and health-care norms in developed countries have shifted the locus of care from institutions to communities[1,2]. Social and health-policy changes have also placed greater emphasis on home and family-care for the chronically mentally ill in these countries. In contrast, families have always been the mainstays of care for the mentally ill in developing countries[3,4].

Family-caregiving is a term used for unpaid care provided by family members or friends to chronically ill or functionally impaired persons[1,5]. The amount of assistance provided by the family-caregiver usually exceeds the level of help provided under ordinary circumstances. Not only is the majority of informal care provided by family members, but the majority of family-caregiving is also carried out by women[6,7]. All over the world, women are the predominant providers of informal care for family members with chronic medical conditions or disabilities, including the elderly and those with other mental illnesses[6-14]. Family-caregiving still remains a predominantly feminine activity despite the fact that with changing demographics and changes in social structures and norms, men are increasingly assuming roles as caregivers[15]. While providing care may have its rewards for family-caregivers, it often entails bearing emotional, physical, social and financial burden, which makes the experience stressful. Despite the voluminous amount of literature on family-caregiving, there is much that remains to be understood about why people take on strenuous caregiving duties, how they approach their caregiving responsibilities, and the consequences of taking up the role of a caregiver[16,17]. For an improved grasp of the experience of caregiving, a more accurate understanding of the caregiving-context which includes gender, familial relationship and cultural background of the caregiver is required[16]. Among these contextual factors, the impact of gender on caregiving has attracted the maximum research attention. The bulk of this research has been carried out among family-caregivers of the elderly with dementia or physical conditions, while gender differences among caregivers of other mental illnesses have been relatively neglected. However, even in this body of research there is considerable disagreement about the exact nature of gender differences in caregiving, and no consistent explanations about how gender influences caregiving.

GENDER DIFFERENCES IN FAMILY-CAREGIVING AMONG THE ELDERLY

Research on gender differences among caregivers of the elderly with dementia and physical illnesses have brought to the fore several themes of interest.

Women predominate among caregivers of the elderly

Worldwide, nearly 70% to 80% of the impaired elderly are cared for at home by their family members[5-7,9,11,17-20]. Varying estimates across different countries indicate that 57% to 81% of all caregivers of the elderly are women[1,6-8,10,12-14,17-26]. In most cases female caregivers are wives or adult daughters of the elderly person. They are usually middle-aged, with a considerable proportion of them being over 65 years themselves. They are also more likely to be employed outside home than in the past[7,17,19,27]. The elderly recipients of care are either frail or chronically physically ill; the majority, however, have dementia or other forms of mental illnesses[7]. Despite the preponderance of women, increased life expectancy, more women working outside home, and smaller families have all increased the pressures on men to assume roles as caregivers of the elderly. Studies in the eighties in the United States suggested that though women predominated as caregivers, somewhere between 20% and 33% of the caregivers of the elderly were men[6,18,19,26]. More recently, it has been reported that the proportion of

men providing care for the elderly has been steadily increasing, so much so that men may constitute nearly half of the primary caregivers of the elderly[8,15,16,19,23,28-35]. Despite the increasing emergence of men as caregivers, research has not taken into account this trend and continues to maintain its traditional focus on female caregivers. Although it appears that men approach caregiving differently, the experience of caregiving among men has not been examined as often as it has been among women[8,15,23,26,29-34].

Gender differences in the experience of caregiving

A number of studies have suggested that the experience of caregiving differs among men and women. Gender-specific differences in the provision of care for those with dementia or physical illnesses have been found to exist in several areas.

Time spent on caregiving and the duration of caregiving: Gender-differences in the time spent on caregiving have been considered in several reviews and studies on the subject. Some of them have concluded that despite conflicting reports, the bulk of the evidence indicates that women devote greater time to caregiving for the elderly, compared to men[1,16,20,25,27-29,34,36,37]. In a comprehensive narrative-review of 30 research-reports, Yee *et al*[22] concluded that the majority of studies which had examined gender differences in the time spent on caregiving had found that women spend more time on caregiving than men. Explanations based on the gendered nature of paid work have argued that women are more likely to care for the elderly because they are less likely to be employed outside home[38]. Women's work roles are viewed as being centred in the home and may reflect a greater sense of family obligation among them[11,16,17,23,38,39]. This increases the likelihood of women spending more time providing care. Time-intensive care among women is also more likely in those societies and cultures, which endorse the traditional value of the woman as the natural caregiver[8,11,23]. However, research findings about gender differences in the time spent on caregiving have not always been consistent. A number of reviews and studies have not found gender to be a significant predictor of the time spent on caregiving[8,10,18,26,38,40-42]. In particular, two meta-analytic reviews on the subject, one of which included 229 studies, have concluded that though women spent more time on caregiving, differences between men and women in this regard were small and of doubtful practical significance[24,43]. There is also considerable agreement that gender differences in the time spent on caregiving are confounded by several other variables such as kinship (spouses *vs* children), and cultural or ethnic influences[10,22,24,26,38,42,43]. Regarding the duration of caregiving, there is far greater consensus that gender does not have an impact on total duration of caregiving[8,10,24,43].

Types of tasks: The literature on gender differences in the type of caregiving tasks has also yielded conflicting findings. A distinction has been made in this literature between tasks associated with personal care such as bathing, dressing and managing incontinence, and tasks associated with management of everyday living. Some studies have found that women are more likely than men to provide assistance with tasks related to personal care[18,44], while others have not reported similar gender differences[9,10,38]. Reviews on the subject have also concluded that gender differences in the types of tasks have only been reported in some but not all studies, and only for tasks related to personal-care. Female caregivers are more likely than men to carry out these tasks[16,17,22,27-29]. Gender differences have not been found in tasks associated with everyday living[38]. These conclusions were endorsed by two meta-analytic reviews[24,43], but these further concluded that gender differences in personal-care tasks were small in magnitude. Gender differences in the types of tasks also appear to be influenced by several mediating variables such as the patient's gender and disability levels, kinship, caregivers' marital and employment status, family composition, social class, and race or ethnicity[10,22,27,38,45].

Role-strain and role-conflict: Caregiver role-conflicts refer to the perceived difficulties in fulfilling the caregiver-role, and the negative consequences emanating from this role[36]. Female caregivers often have to play multiple roles such as wives, daughters, mothers, or employees[16,17,38]. The pressures of enacting these conflicting roles may create difficulties for women. Role-conflicts and role-strains may manifest in many ways[16,17,38,46]. Role-conflicts arise when conflicting and incompatible demands are made of the caregiver himself/herself[16,17]. Role-strain occurs when one is unable to meet the expectations and obligations of multiple roles. Role-overload sets in when these competing demands overwhelm the person's ability to carry out his/her role[16,17]. This might lead to role-captivity, which refers to the caregivers' feelings of being trapped in their roles[17,46]. Role-conflicts give rise to several adverse consequences for caregivers such as physical problems, fatigue, burnout, depression and other emotional disturbances, and feelings of resentment towards the patient[17,46]. Many studies have found that female caregivers of the elderly with physical problems or dementia experience greater role-strain and role-conflict than male caregivers[1,13,14,16,20,27-29,36,39,44,47-52]. Women appear to experience greater interference and limitations in their work and social life because of their role as caregivers. They are generally believed to experience greater role-strain due to the more intense care they provide. Greater role-strain in women produces more frequent health problems, a less positive outlook on life, and a greater need for external support. From their review of nine studies on gender differences in caregiving role-strain, Yee *et al*[22] concluded that

female caregivers report that their caregiving-roles interfered with their work and social life to a greater extent than men. However, such findings have not always been consistent, with several studies finding that caregivers' gender has no impact on their evaluations of role-strain[20,32,42,53]. It has been suggested that differences between studies arise more from the fact that perceptions of role-strain may vary depending on whether the caregivers are spouses or children of the elderly[36,44,47,50,54].

Satisfaction with caregiving: There is a relatively small amount of research-data on gender differences in other aspects of caregiving such as satisfaction with caregiving. The findings are equivocal, with some studies reporting that women are less satisfied[55-58], while a similar number of studies have found no differences in satisfaction between male and female caregivers[10,33,59,60].

Reasons for providing care: Several authors have identified emotional and social connectedness of women towards their patients, as well as their sense of family obligation as the basis for their nurturing approach to caregiving[16,61,62]. Women appear to be more concerned about the emotional well-being of the people they provide care for. This attachment often motivates them to engage in caregiving[20,38,61-63]. A greater sense of responsibility towards the patient, altruism, and self-sacrifice has also been found to characterize women's attitudes to providing care[10,25,27,33,38,45,61-63]. However, studies of male caregivers have suggested that caregiving among men is also driven by a similar sense of affection, commitment, and family responsibility[27,30].

Gender differences in caregiver-burden

Caregiver-burden has been defined as "a multidimensional response to physical, psychological, emotional, social, and financial stressors associated with the caregiving experience"[5,64,65]. Caregiver-burden is often the final outcome of a stressful and negatively perceived experience of providing care[66]. Not surprisingly, the greater part of the literature on gender differences in caregiving has been devoted to the subject of caregiver-burden. However, the results have been far from conclusive. Though a number of studies have found that female caregivers report greater levels of both objective and subjective burden[8,10,11,17,23,33,34,39,44,46-48,51,65,67-73], a similar number have been unable to find any gender differences in caregiver-burden[9,12,30-32,36,50,53,54,74-83]. Moreover, some studies have found differences in only certain aspects of burden, *e.g.,* subjective burden, and not in others[8,10,17]. Narrative reviews on the subject have been similarly uncertain in their conclusions. While some of them have concluded that caregiver-burden is higher among female caregivers[5,22,25,27,29,84-86], others have not found evidence in favour of greater levels of burden among women[12,15,30]. In their seminal narrative-review on gender differences in caregiving, Yee *et al*[22] extracted data on caregiver-burden from 17 of the 30 studies they had included in their review. The vast majority of these studies reported that women experienced higher levels of caregiver-burden than men. However, meta-analytic studies have come to somewhat differing conclusions. An early meta-analysis included 14 studies on caregiver stressors and burden among the frail elderly[43]. It found that though female caregivers were more likely to report greater caregiver-burden, differences between the genders were small. In another meta-analysis of 4 studies, female caregivers of patients with dementia reported poorer global self-health, but did not differ from male caregivers on other risk factors[52]. In a meta-analysis of 84 studies of caregivers of the frail elderly, Pinquart *et al*[87] found that higher stress and poorer well-being among caregivers were more common among older women who were spouses of the patients. However, a later meta-analysis of 176 studies of caregivers of the elderly by the same authors found that associations of caregiving stressors with health were stronger among older men proving care for those with dementia[88]. The same authors have also carried out the most comprehensive meta-analysis till date of 229 studies on gender differences in caregiving of the elderly[24]. In this meta-analysis, the authors found that female caregivers had higher levels of burden and lower levels of subjective well-being and physical health compared with men, but these differences were small and barely reached the threshold of practical significance. Thus, they concluded that the available evidence indicated that there are more similarities than differences between male and female caregivers in this regard, and that some of the apparent gender differences could have arisen from methodological variations, or the effect of other confounding factors on caregiver-burden[24].

Gender differences in psychological morbidity

In their review, Yee *et al*[22] found nine studies which had examined gender differences in depression among caregivers of the frail elderly, and three studies which had reported gender differences in general psychiatric symptomatology. Overall, in 10 out of these 12 studies higher levels of depression and psychological morbidity was reported among female caregivers. Other reviewers have also reported greater psychological morbidity, principally depression, among female caregivers of the elderly[20,27,34,85,89,90]. Additionally, gender differences in psychological morbidity have been found in other studies[35,48,67,69,71-73,91]. In contrast, several studies have not been able to find significant differences among male and female caregivers in depression or psychiatric symptom-scores[23,33,75-78,82,92]. Meta-analytic reviews, though finding a higher prevalence of depression among female caregivers of the elderly, have reported that these gender differences were of much smaller magnitude than expected[10,24,43,52].

GENDER DIFFERENCES IN FAMILY-CAREGIVING AMONG SCHIZOPHRENIA AND MOOD DISORDERS

The issue of gender differences in family-caregiving in schizophrenia and mood disorders, or other psychiatric conditions has not been examined as comprehensively as among the elderly. Studies, which have evaluated burden among caregivers of such illnesses, have only occasionally considered gender of the caregiver when examining the numerous correlates of caregiver-burden. Nevertheless, certain trends similar to the literature on gender differences in the elderly are still evident.

Gender and type of caregivers of patients with schizophrenia and mood disorders

In a recent review of 42 studies on caregiver-burden in schizophrenia, the majority of caregivers were mainly the parents (usually mothers), followed by spouses and siblings of patients[93]. In an earlier review, Awad *et al*[94] had reported that women, either wives or sisters formed the greater part of caregivers of those with schizophrenia. They quoted a United States community survey, in which women constituted 82% of caregivers, with 90% being mothers of patients; 70% of them were over 60 years of age. This trend has been endorsed by a number of other reviews, which show that most family-caregivers of those with schizophrenia are their parents, mostly mothers of patients, and they are usually elderly[95-101]. However, the number of male caregivers seems to be on the increase[94,98,102], while in certain cultures men often predominate as caregivers[93,98,103,104].

Gender differences in caregiver-burden and psychological distress

Not only is there limited research on gender differences in caregiver-burden among schizophrenia and mood disorders, but the evidence for such differences is also less obvious. In their review of caregiver-burden in schizophrenia, Caqueo-Urízar *et al*[93] noted that female gender, unemployment and time spent in caregiving were all associated with higher burden. In contrast, in an earlier review of patients with severe mental illnesses other than dementia, Baronet[101] had identified 10 studies, which had evaluated the relationship between burden and caregivers' gender. None of them had found gender differences in overall burden, objective burden, subjective burden, worry, fear, or stigma. The results of individual studies conducted among family-caregivers of those with schizophrenia have also varied considerably. A number of these studies have reported higher levels of caregiver-burden, stress, burnout, psychological morbidity and poorer quality of life among female caregivers of those with schizophrenia[97,100,102-114]. However, several other studies have not found any differences in caregiver-burden between the genders[98,115-124]. Then again, very few of these studies on schizophrenia have actually conducted comprehensive examinations of gender differences among caregivers. In an Indian study, caregiver-burden was examined in 70 spousal caregivers of patients with schizophrenia. Results showed that female spouses experienced significantly greater total burden and burden in the areas of external support, caregivers' routine, patients' support, patients' behaviour, and caregivers' coping strategies[106]. Female spouses also felt more anxious, tired, frustrated or isolated, and had to face a greater work load. Another study examined differences in caregiving between mothers and fathers who had a son or daughter with schizophrenia, in 100 such caregiver-couples[115]. The results showed that men and women were equally vulnerable to caregiving stressors. Studies among caregivers of patients with bipolar disorder are fewer. Perlick *et al*[125] examined gender differences among 150 primary caregivers of patients enrolled in the Systematic Treatment Enhancement Program for Bipolar Disorder. They found that men and women did not differ on depression or caregiver-strain. Some of the other studies of bipolar disorder have found higher levels of caregiver-burden or poorer quality of life among female caregivers[111,126,127], while others have not[128]. In a study of depressed patients with both unipolar and bipolar depression, wives were found to be more isolated and upset compared to their husbands[129]. However, results of other studies on depressive disorders have been mixed, with some reporting higher caregiver-burden or greater levels of depression among female caregivers[97,130,131], while others have not found significant gender differences in either burden or psychological morbidity[128,132]. In a recent study, a comprehensive examination of burden, psychological morbidity and other caregiving-indices was undertaken among male and female caregivers of 100 Indian patients with schizophrenia and recurrent mood disorders[133]. The majority of female caregivers were housewives. Male caregivers were more likely to be in paid employment than the female caregivers, had significantly higher income and were more likely to belong to the upper socio-economic strata than female caregivers. A significant gender difference emerged in the time spent on caregiving, with female caregivers spending more time providing care for their patients. Female caregivers also scored significantly higher in one domain of negative appraisal, while male caregivers had significantly higher scores on family-cohesion. Men cited family tradition, familial obligation, and concern about the patient's ill health as their reasons for providing care more often than women. Women, on the other hand, were more likely than men to report dependence, especially socio-economic dependence on their male patients, feelings of affection and sympathy for them, and a greater concern about the patient's future as their reasons for providing care. However, there were no significant differences between male and female caregivers in any of the areas of objective or subjective burden, or psychological morbidity. Moreover, there

were no differences in coping strategies, availability of social support, or personality traits such as neuroticism and rumination between men and women. The correlates of caregiver-burden and distress were largely similar among male and female caregivers. Finally, multivariate analyses showed that caregiver-gender explained only a minor proportion of the variance in caregiver-burden and distress. The results of this study thus endorsed what appears to be the consensus view in literature, largely derived from research among the elderly, that gender differences in caregiving though present are minor in nature, and caregiver-gender explains only a very small proportion of the variance in caregiver-burden and distress.

GENDER DIFFERENCES IN FAMILY-CAREGIVING: PROBABLE EXPLANATIONS

Several theories have been advanced to explain gender differences among caregivers of the elderly. Sociological explanations have emphasized expectations of traditional gender roles, in which women are expected to adopt the role of a caregiver. This is ingrained in females through their social and cultural experiences starting from childhood, and leads to a different approach to caregiving compared to men. Additionally, theories of segregation of labour indicate that since women are more likely to stay at home it is natural for them to take up the caregiver role[11,16,17,24,36, 38,39,45]. Men are not traditionally expected to become caregivers, which leads to a dissimilar approach towards caregiving among them. Due to their role-socialization, men may also be less adept at expressing their difficulties or emotions. This could result in a response-bias, in which men may be less likely to report difficulties in providing care than women[8-11,23,27,72]. However, it has been noted that empirical support for these theories is lacking[10,17,24,43,52]. Therefore, a second set of explanations based on the "stress-coping" theory has been proposed. It has been argued that gender differences arise because female caregivers have greater exposure to caregiving stressors, and differ in their appraisal, coping and availability of social support while managing these demands[10,11,17,22,24,65]. Though this theory has found more support from different studies, but unequivocal evidence of gender differences in appraisal, coping and social support is also lacking[8-11,39,22-24,27,48,68,72,73,134]. Some studies have indicated that gender differences in caregiver-burden and distress could be due to a differentiated appraisal of the caregiving situation among men and women[10,73], but the evidence for such gender differences is limited. Gender differences in coping strategies have been examined more extensively. Among family-caregivers of the elderly, a number of studies have found that women use emotion-focused coping and other ineffective coping styles such as fantasy, wishful thinking denial, escape, or avoidance, more frequently than men. In contrast, men have a wider coping repertoire than women, and use more effective coping strategies such as problem-solving, acceptance, detachment or distancing more frequently[11,25,48,72,77,134]. These differences in coping strategies could potentially explain the higher levels of caregiver-burden and psychological morbidity among women[8,11,22,23,48]. Similar gender differences in coping have occasionally been reported among caregivers of patients with schizophrenia and mood disorders[106,135,136]. However, the number of such studies is limited, both among the elderly, as well as in schizophrenia and mood disorders. Contrary findings of lack of differences in coping between male and female caregivers have also been reported[22,73]. Some authors have proposed that female caregivers experience higher burden and distress because of lack of available social support[8,16,22,67]. According to them, women who care for the elderly are less likely to seek or receive support, because of the restrictions imposed on them by their caregiving roles. Men, on the other hand, are more inclined to seek and receive outside help for caregiving from formal and informal sources. Women seem to have larger social networks and more available sources of informal support, while men who have less access to formal and informal support, may be more motivated to seek help from these sources[22,24,137]. A greater lack of social support among women has also been found in spouses of patients with schizophrenia or mood disorders[106,129]. Again, the available results evidence are inconsistent in this regard, and gender differences in social support are not as pronounced as expected[10,22,24], either among caregivers of elderly persons or those with other psychiatric illnesses. Neuroticism, the greater propensity to break down under stress has been shown to have a significant influence on burden and psychological morbidity among caregivers. Some of the evidence indicates that the higher levels of depression and psychiatric symptoms among female caregivers could be partly accounted for by their higher neuroticism and greater use of escape-avoidance coping, but the number of such studies is small[22,46,72].

Since gender differences in appraisal, coping, social support and personality traits have been minimal and inconsistent, other explanations have been sought to account for differences in family-caregiving between men and women. It has been argued that the impact of gender is mediated by several other variables. These include characteristics of the patients, the severity of their illnesses including behavioural problems and associated disabilities, composition of the family, caregivers' demographics such as age, marital status, education, employment and socioeconomic status, their relationship with the patient, and the effects of culture, and ethnicity[5,8-11,22-28,34,38,39,44,46,50,67,68,73,79,86]. The influence of culture and ethnicity and kinship with the patient and has been explored in a number of studies. It is an undisputed fact that culture and ethnicity have a seminal influence on caregiving[138]. However, whether

cultural and ethnic factors impact gender differences in caregiving is a matter of some dispute. Certain authors have stated that studies from various cultures generally find that female caregivers are at greatest risk for caregiver-burden[5,93]. Others have proposed that gender differences in caregiving are less likely in cultural and ethnic groups with more positive attitudes towards the elderly, a traditional emphasis on women as caregivers, and the relative unavailability of formal sources of care[8-11,13,14,38,45,50,73,79,139]. It has also been suggested that among certain cultural or ethnic groups, familial-cultural variables such as familism, family-support, filial responsibility and family-cohesion may contribute to the gender differences in caregiving[5,8,10,11,73]. Familism refers to the precedence given to the family needs over the needs of the individual, while family-cohesion refers to the emotional bonding that family members have towards one another, and filial responsibility or piety refers to the tradition of caring for one's elders[5,140]. However, the exact direction of gender differences due to familial-cultural variables is unclear, because both higher burden among female caregivers[5,8,10,11,73], or similar levels of burden between the two genders[12,79,133,141] has been reported among cultural or ethnic groups with these familial-cultural values. Kinship status of the primary caregiver is another factor, which is thought to have a significant bearing on gender differences in caregiving[5,20,22,24,26,27,34,39,45,46,54,65,86]. Many studies have found greater burden or strain among spouses (usually wives) than children[9,11,23,38,79]; others have found the obverse[39,47,67], while some have found no effect of kinship ties on gender differences among caregivers[50,74].

GENDER DIFFERENCES IN CAREGIVING: METHODOLOGICAL VARIATIONS

Methodological variables contribute a great deal to the observed gender differences[15,24,29,86,101]. It has been repeatedly pointed out that there is a great deal of difference across studies in their samples, designs, assessment-procedures, data analyses and theoretical frameworks. These methodological variations could account for a large proportion of the variance in findings, and may give rise apparent rather actual differences between male and female caregivers[1,15,22-24,27,29,36,43,52,85,86,89,101].

GENDER DIFFERENCES IN CAREGIVING: CONCLUSIONS AND FUTURE DIRECTIONS

Across the world women still constitute the majority of caregivers either of the elderly, or of those with other psychiatric disorders. However, the proportion of men taking up the caregiver's role is steadily increasing. Although a large body of the evidence seems to indicate that women suffer more from the negative consequences of providing care, several other trends apparent in research need to be noted. Despite extensive examination of the area, gender differences in caregiving have not been consistently or conclusively documented. The magnitude and significance of the gender differences, which have been found is also uncertain. The majority of studies have been carried out among women; the experience of male caregivers has been relatively neglected. The bulk of the evidence comes from studies conducted among the elderly; gender differences in conditions such as schizophrenia or mood disorders have not been examined as comprehensively. Many explanations have been provided for greater burden and distress among female caregivers, but most of them are not supported by data. The effect of several variables, which mediate the influence of gender on outcomes of caregiving is uncertain. Finally, methodological variations between studies may conceal the true nature and extent of gender differences. Future research will need to address all these deficiencies before a better understanding of the subject can be obtained. If significant gender differences are indeed found, these will have major implications for development of gender-specific caregiver interventions, and social policy recommendations to improve the plight of female caregivers. It is for this very reason that there is much scope for further research in this area.

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REVIEW

Neuronal networks in mental diseases and neuropathic pain: Beyond brain derived neurotrophic factor and collapsin response mediator proteins

Tam T Quach, Jessica K Lerch, Jerome Honnorat, Rajesh Khanna, Anne-Marie Duchemin

Tam T Quach, Jessica K Lerch, Department of Neuroscience, Center for Brain and Spinal Cord Repair, the Ohio State University, Columbus, OH 43210, United States

Tam T Quach, Jerome Honnorat, Lyon Neuroscience Research Center, INSERM U1028/CNRS UMR 5292, F-69372 Lyon, France

Jerome Honnorat, French Reference Center on Paraneoplastic Neurological Syndrome, Hospices Civils de Lyon, Hôpital Neurologique, Neurologie B, F-69677 Bron, France

Jerome Honnorat, Université de Lyon, Université Claude Bernard Lyon 1, F-69372 Lyon, France

Rajesh Khanna, Department of Pharmacology, College of Medicine, the University of Arizona, Tucson, AZ 85724, United States

Anne-Marie Duchemin, Department of Psychiatry, College of Medicine, the Ohio State University, Columbus, OH 43210, United States

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Correspondence to: Tam T Quach, PhD, Department of Neuroscience, Center for Brain and Spinal Cord Repair, the Ohio State University, 460 12th Avenue, Room 696, Columbus, OH 43210, United States. quach.1@osu.edu

Telephone: +1-614-2921887
Fax: +1-614-2937599

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Abstract

The brain is a complex network system that has the capacity to support emotion, thought, action, learning and memory, and is characterized by constant activity, constant structural remodeling, and constant attempt to compensate for this remodeling. The basic insight that emerges from complex network organization is that substantively different networks can share common key organizational principles. Moreover, the interdependence of network organization and behavior has been successfully demonstrated for several specific tasks. From this viewpoint, increasing experimental/clinical observations suggest that mental disorders are neural network disorders. On one hand, single psychiatric disorders arise from multiple, multifactorial molecular and cellular structural/functional alterations spreading throughout local/global circuits leading to multifaceted and heterogeneous clinical symptoms. On the other hand, various mental diseases may share functional deficits across the same neural circuit as reflected in the overlap of symptoms throughout clinical diagnoses. An integrated framework including experimental measures and clinical observations will be necessary to formulate a coherent and comprehensive understanding of how neural connectivity mediates and constraints the phenotypic expression of psychiatric disorders.

Key words: Neuron; Network; Synapse; Schizophrenia; Bipolar; Depression; Stress; Pain; Collapsin response mediator proteins

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Core tip: Increasing evidences suggest that mental diseases are neural network disorders. Neurites and synapses represent the sub-cellular elements organizing these networks, and the molecules that regulate their formation, retraction and adaptive remodeling may contribute to the pathology of mental disorders. Various syndromes may share alterations of functional network leading to symptoms overlapping through clinical diagnoses.

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INTRODUCTION

Despite their great diversity of morphology, most vertebrate nerve cells exhibit distinctive polarized structures with a single long axon and a specific dendritic arbor depending on their location. The axon/dendrite identity that influences the synaptic genesis and inputs that each neuron can integrate emerges as a convergent product of specific pattern of growth, branching and retraction and is differentially regulated at multiple points, including the control of the number of primary branches and their mode and frequency of branching by inhibitory (*e.g.,* Sema3, Nogo-A) and permissive [*e.g.,* brain derived neurotrophic factor (BDNF); fibroblast growth factor (FGF)][1-4] factors. Moreover, neurons do not connect indiscriminately between themselves but form an intricate non-random highly selective short [the hippocampal-prefrontal cortex (HIP-PFC) or hippocampal-amygdala-prefrontal cortex circuits][5,6] to long (the corpus callosum that connects the two cerebral hemispheres -facilitating an array of connective function- and influences higher cognition as well) range white matter axon fibers connections[7] producing characteristic networks capable of ensuring proper healthy behavior. The neuronal structure and neurotransmission in these networks, developed by interactions with the environment, are constantly remodeled *via* "activity-dependent synaptic plasticity"[8] to process, store and transmit relevant information. Accordingly, abnormal changes in the production of neurotrophic factors and permissive or inhibitory guidance cues may induce structural and/or functional abnormalities which may alter information processing and consequently link neuronal network formation/maintenance to mental disease(s). Since psychiatric disorders have overlapping symptoms - *e.g.,* cognitive impairment and emotional dysregulation that can be found in schizophrenia, depression and anxiety disorders it is likely that this similarity is the consequence of disruption in common brain circuits (*e.g.,* hippocampus-prefrontal cortex) over time. In this review, we provide new molecular and cellular insights detailed from gene targeting/genome-wide association data (GWAS)[9,10] that extend the understanding of mental diseases and improve their treatment, to network theories that highlight structural and functional brain connectivity. Structural connectivity corresponds to the anatomical neurites, synapses-connections between neural elements whereas the functional and effective connectivity refers to a statistical dependence between the physiological signals measured in each region and the influence that one region exerts over another respectively. We focus on two brain areas essential in the emotional and cognitive domains: The hippocampal formation (hipF) and the prefrontal cortex. Their functional coupling support multiple functions, including emotion, mood, memory, thinking about the past and future, self and others, which are altered in mental diseases.

MOLECULAR BASIS OF NEURONAL NETWORK GENESIS AND MAINTENANCE

Neurogenesis and neuritogenesis

The generation of neurons (neurogenesis) in various regions of the central nervous system depends on a carefully regulated process of neural progenitor cells proliferation and differentiation. During early development, the neural tube wall constitutes a pseudo-stratified epithelium made of highly polarized neuroepithelial cells. The proper amount of neurons is spatially and temporally controlled by accumulative activities of numerous extra-/intra-cellular factors. At the onset of neurogenesis and initiated by extracellular regulators (Notch, bone morphogenetic proteins, BDNF/TrKB/p75, Wnt/β-catenin/Sonic hedgehog)[11-13] and intracytoplasmic transcription factors (bHLH, Insm1, AP2γ, TRIM32)[14-16], these cells switch their identity and turn into radial glial cells (RG) expressing glutamate-aspartate transporter and brain-lipid binding protein[17]. They generate all diverse intermediate progenitor neurons and glial cells through orchestrations of numerous molecules (Nrg1, Foxg1, Retinoid acid)[18-20] and signaling mechanisms (Jack/Stat, Notch, BMP, FGF)[21-23]. Shortly after, the pro-neural factors (Ngn1/Ngn2/Ascl1, also known as Mash1)[24,25] and transcription factors (Salb2, Sima1Am2, Lhx2)[26-28] activate a generic program of neurogenesis, arrest the division of progenitor cells, suppress the alternative astroglial fate and select the neuronal fate. Subsequently, the newborn neurons extend neurites (neuritogenesis which begins with the concerted accumulation and organization of actin/microtubules)

and migrate to appropriate locations. However, due to heterogeneous neuronal phenotypes generated at different times, in different layers and locations, there is considerable heterogeneity in neuritogenesis mechanisms. This makes it difficult to observe *in vivo* although we found recently that collapsin-response-mediator-protein (CRMP)3[29] has a profound influence on lamellopodia formation, neuritogenesis and dendritic arborization in vertebrate hippocampal neurons: The CRMP3$^{-/-}$ knock-out mice[30] display abnormal functional and structural neural networks associated with a delay in neurite outgrowth and alterations of dendrites and spines but not axon-morphology in hippocampal neurons during development that persist in adults. These alterations affect a subset of hippocampal circuits and hippocampal function: CRMP3$^{-/-}$ mice have a deficit in prepulse inhibition found in several mental diseases and abnormal long term potentiation (LTP). Such a bona fide mouse model is a critical first step towards exploring pathogenic mechanisms. There is increasing evidence that other CRMPs are involved in neurogenesis in the adult dentate gyrus and the olfactory system, and also regulate dendritic/axonal outgrowth[31-33]. Specifically, required for NT3-induced axon outgrowth/branching and linking kinesin to the Sra-1/WAVE cargo complex in axons, CRMP2 can convert established dendrites to axon or induce supernumerary axons[34] while mice lacking individual CRMP1, 4 or 5 present alterations in neuronal differentiation of specific brain areas and the behavior they control[35-37].

Another way to investigate neuritogenesis is using primary hippocampal neurons in culture. Few hours after plating, neurons start to extend minor neurites; then one of the multiple neurites extends rapidly and by morphological transformation generates the axon through stochastic selection; the other neurites mature into dendrites, leading to neuronal polarity. Live cell imaging using time-lapse video microscopy shows that the first two neurites have the highest potential to become axon. Because cultured neurons develop polarity without any presence of exogenous extracellular guidance/neurotropic cues, it has been suggested that an internal polarization program exists involving several intra-neuronal organelles and distinct repertoires of signaling molecules intrinsic to the neuron. Using that model, we reported that whereas neurons from heterozygous CRMP3$^{+/-}$ mice polarized and grew similarly to control wild type (WT), all CRMP3$^{-/-}$ neurons from homozygous CRMP3$^{-/-}$ littermates did not establish neuronal polarity. Such impairment in neurites to progress from stage 1 to stage 2 represents a failure of neurite initiation. Moreover, the correlation between the levels of CRMP3 expression and its activation of L- and N-type of voltage-gated calcium channels suggests that the facilitatory role of CRMP3 on neurite initiation, dendritic development and plasticity may be mediated *via* Ca^{2+} influx[38]. Other factors such as neurotrophins, extracellular matrix proteins, attractant/repellent guidance cues and guide-post proteins are considered extrinsic signals. Interactions between these molecules can provide short/long range guidance information or stably change the intrinsic ability of a neuron to extend/retract neurites during development or to engage them into an axono/dendritic differentiation path. It is tempting to suggest that similar players are required to refine neuritogenesis and to make synaptic connections (synaptogenesis) *in vivo* within the developing brain.

Synaptogenesis and neuronal network genesis

Brain complexity comes from the large diversity and number of neurons and the variety and number of synapses where neurons transfer their electrical and/or chemical signals to other neurons/cells. Electrical and chemical synapses differ in the molecular mechanisms supporting the transmission of information and in their morphological organization[39,40]. Their structure and composition vary across brain regions and their disruption in function and morphology may be involved in many neurological/mental diseases and after trauma. Electrical synapses are also a prerequisite for the chemical synapses formation in mammal brain during development. Within electrical synapses the gap junction channels processed by connexins and pannexins serve as conduits allowing a direct bidirectional communication and passage/exchange of metabolites, intracytoplasmic messengers, and ions between the cytoplasm of two cells (Figure 1B). Within neuronal networks, these electrical synapses provide synchronous electrical activity and field potential oscillations. They mediate an important form of direct intercellular communication and allow rapid transfers of pre-synaptic excitatory electrical impulses to post-synaptic potentials throughout the intercellular gap by generating synchronous oscillations of gamma-frequency (30-70 Hz) rhythms important for field potential oscillation within neuronal networks and necessary for the interplay of neural populations involved in memory processes.

In contrast, there is no cytoplasmic junction between two cells at the two chemical synaptic subtypes: The excitatory asymmetric (mainly glutamatergic; Figure 1A) type Ⅰ synapse has marked postsynaptic densities (PSD) while the inhibitory symmetric (mainly GABAergic) type Ⅱ synapse has no thickened PSD. The genesis of chemical synapses is characterized by an enormous degree of complexity and diversity of protein-protein interactions. Axonal presynaptic boutons of excitatory synapses contain round clear vesicles loaded with the neurotransmitter glutamate and connected with dendritic spines while inhibitory presynaptic boutons contain slightly smaller vesicles and are most abundant at the neuronal soma. In CNS, maturation and stabilization of synaptic structures depend on neurexins and neurolignins, the molecule pairs in the CAM family[41-43], while plasticity which allows an individual to adapt to a rapid changing environment through strengthening, weakening, pruning or adding synaptic connection-is partially dependent on BDNF, ephrin, Wnts, NgR1, semaphorins class 3 and non-coding RNAs[44-48]. The

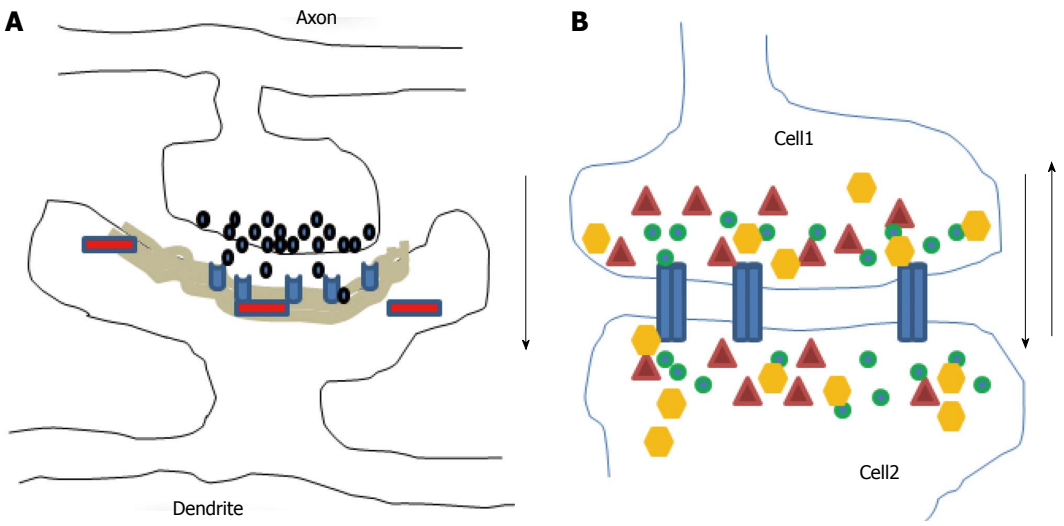

Figure 1 Diagrams illustrate chemical (A) and electrical (B) synapses. At chemical synapses, neurotransmitters (black) released from axonal boutons bind to postsynaptic receptors (light blue) and trigger specific signaling pathways *via* activation of proteins (red) in postsynaptic cells with prominent postsynaptic densities component (grey area). The information transmission is unidirectional (black arrow). At electrical synapses, gap junction channels (blue) directly connect the two adjacent cells, thus enable the bidirectional passage of electrical currents (black arrows) carried by ions (green), and of small peptides (yellow) or second messengers (dark red).

mechanisms contributing to the synaptic plasticity include structural remodeling of the synapse, structural reorganization of presynaptic active zone, postsynaptic density area, protein synthesis, signal transduction pathways, Ca^{2+} fluxes, kinases activities, neuronal activities, change in transmitters release and receptors trafficking. However, plasticity creates a significant challenge to the intrinsic architecture and integrity of neural networks which is counterbalanced by compensatory regulatory mechanisms for maintaining neuronal homeostasis, leading to a balance between wiring plasticity and stability.

Synapses, the sites where two neurons connect and pass information, are the building blocks of the neuronal networks defined by "a set of elements with time-variant properties that interact with each other" and network dynamics defined by the real-time changes in response to internal and external stimuli. It should be noted however that information is not stored in a chemical form but is processed and retrieved by neuronal networks. The global neuronal network complexity can be defined as a dynamic interconnected functional system characterized by a series of simpler networks organized into increasingly effective local or global complex networks constrained however by the intrinsic structural brain architecture[49]. It has the capacity to support complex thought, action and learned behavior that any single neuron/element of the system would not be able to support alone and its resting-state reflects the stable and intrinsic functional architecture of the brain. Importantly, dysfunction of local networks may spread easily between linked elements, leading to pathological cascades that cause dedifferentiation or trans-neuronal degeneration and encompass large areas of the system. It may also lead to dynamic adjustments and reorganization of the other networks to compensate these changes. Consistent with the view that network organization fundamentally influences brain diseases, many studies including connectomic approach, address the behavioral impairments that arise from network insults or dysfunction and challenge to predict patterns of disease spread and targets of intervention[50,51]. In various mental diseases, neuroimaging observations, diffusion tensor imaging, electroencephalography (EEG) and magnetoencephalography report altered structures and functions of PFC and hipF and deficits in functional integration between these two elements (Figure 2) suggesting overlapping pathogenic mechanisms.

The PFC, which matures later in development than more caudal cortical regions, exerts "top-down" control of many cortical and sub-cortical areas; moreover some of its neuronal subpopulations exhibit complex dendritic arbor. Its development is characterized by growth in early childhood, decrease in adolescence and continued maturation in adulthood. It is well established in human that PFC is involved in language, maintenance of attention, executive functioning, organization of inputs from diverse sensory sources, coordination of goal-directed behavior, socialization and moral decisions. HipF is structurally and functionally heterogeneous. The anterior and posterior parts receive/extend different afferent and efferent connections and play a role in various functions such as learning and memory, stress, spatial and emotional processing. The neuronal projection from the HIP either directly -monosynaptic- or indirectly polysynaptic to the PFC is referred to as the hipocampal-PFC pathways (HIP-PFC). In rats, the direct monosynaptic HIP-PFC pathway originating from the CA1 and the subiculum projects to the anterior cingulated areas of the PFC through fimbria/fornix system. It exhibits activity-dependent synaptic plasticity such as LTP/LTD or depotentiation. Treatment with

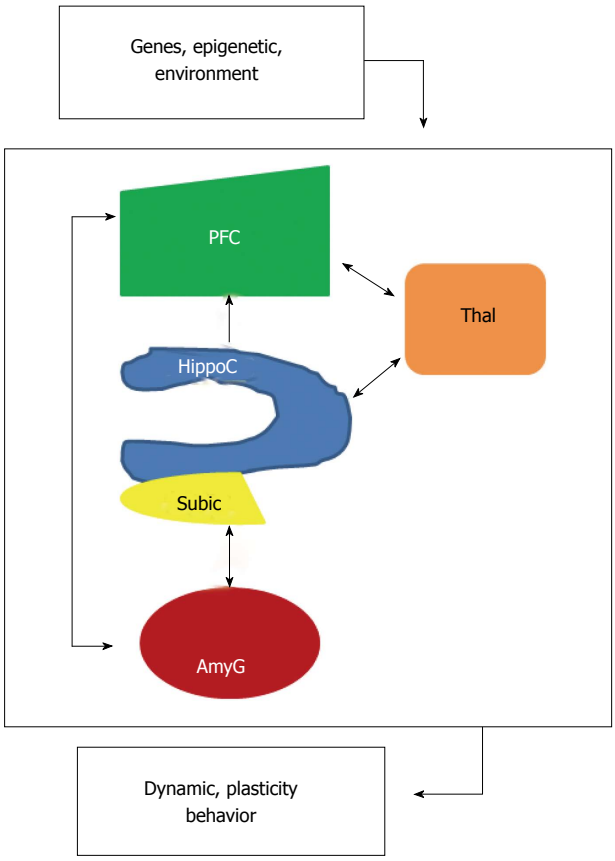

Figure 2 Localized interregional connectivity. Diagram shows pathway connections of hippocampus (blue), amygdala (dark red), thalamus (orange) and prefrontal cortex (green). HippoC-PFC pathway originating from the subiculum and the CA1 of the hippocampus to the PFC is unidirectional, direct in a monosynaptic manner in rodents and primates. HippoC-AmyG pathway shows bidirectional connection between ventral HippoC with AmyG and the pathway AmyG-PFC has also bidirectional connections. Together, both the HippoC and PFC are reciprocally connected with the AmyG and disruption of these pathways, anatomically or functionally may be a common origin of mental diseases. Moreover, there are bidirectional connections between PFC/HippoC with Thalamus. PFC: Prefrontal cortex.

lidocaine disrupts its performance. However, fine details of the human HIP-PFC are lacking because direct powerful tract-tracing techniques cannot be applied. Within the hipF, neuroanatomical studies show that the ventral CA1 and subiculum also project to the basolateral amygdala (AmyG) which has a critical role in expression of fear and autonomic defense responses whereas recent works highlight the importance of distinct AmyG projections to other structures and their importance in controlling reward/learned behavior. Functional imaging in patients with major depressive disorder (MDD) and bipolar disorder (BP) shows that the rate of resting cerebral blood flow and glucose metabolism in AmyG is elevated and positively correlates with depression severity and relapse. In addition, the dual-projection of hippocampal neurons is crucial for coordinating PFC/AmyG activity during memory retrieval. Meanwhile, AmyG neurons densely arborize within superficial layers of PFC and form synapses with layer Ⅱ pyramidal neurons. Disruption of the AmyG-PFC pathway increases choice of risky rewards suggesting that it is important for top-down control of emotion, anxiety and fear. Presently, it is well-established that patients suffering from a number of mental diseases, such as schizophrenia, major depression, bipolar and PTSD, display cognitive impairment, have structural abnormalities, disorganized neural networks and aberrant functional coupling within HIP/PFC/AmyG and their pathways[52-56].

NEURONAL NETWORK ABNORMALITIES IN MENTAL DISEASES

Schizophrenia

Schizophrenia is a complex psychiatric disorder with variable symptomatology characterized by hallucination, delusion, anhedonia and cognitive dysfunction. Studies of post-mortem brains provide patterns of abnormalities that reflect failures in early brain development and maturation which can be attributed to alterations in gene expression (NRG1/Akt/Dysbindin-1/Reelin/COMT/DISC1) affecting neuronal differentiation[57-60], gene duplication (25q11-13, 16p11.2, 16p13.1)[61,62] or deletions (2p53, 3q29, 15q13-q14, 15p11.322q11.2)[63,64] . There is also evidence for contribution of multiple different epigenetic events [stress that activates the hypothalamic-pituitary-adrenal (HPA) axis and increases dopamine brain function, viral infection, DNA methylation, histone modification, non-coding RNAs]. Of note, recent proteomic, gene targeting, post-mortem and SNP linkage studies include CRMP1 and 2 in the list of schizophrenia susceptibility genes[65,66]. As schizophrenia typically starts in the late adolescence and as brain development is a continuous process, the remodeling of cortical and hippocampal structures and synaptic connections is thought to be critical. Indeed, patients with schizophrenia show structural anomalies in the HIP and cortical thinning in the PFC associated with aberrant functional coupling -*i.e.,* reduced fractional anisotropy values in the superior longitudinal fasciculus white matter bundle between two areas during resting state or working memory present in both first-episode patients and persons at risk[67]. This scheme suggests that the dysfunction is not a consequence of the disease or treatment but is consistent with abnormal HIP/PFC interaction. Precisely, it has been proposed that deficit in emotional regulation is likely dependent on the dysfunction of HIP, which contributes to aberrant dopaminergic synaptic activities in nucleus accumbens, which in turn influences PFC maladaptive processes leading to delusion and psychosis. To this extent, structural and functional vulnerability and abnormalities of GABAergic, glutamatergic serotoninergic and cholinergic synapses have been reported by several groups[68,69]. As synapses are ultimately linked to neurotransmitter release and their signal transduction and as spine morphology is closely linked to synaptic function, altered spine shape, size and density have multiple functional effects on neuronal networks, and

dendritic spine dysfunction may have an etiological role in schizophrenia. Several post-mortem studies reveal altered white matter myelination/projection and a profound reduction in spine density in the PFC of patients while the reduction in spine density found in the auditory cortex could potentially be associated with auditory hallucinations. Other evidence suggest that the heterogeneity of schizophrenia may originate from larger disturbances in neural elements of several interconnected brain circuits and structures[70] including HIP, parahippocampal gyrus, entorhinal cortex, AmyG, superior and transverse temporal gyri, prefrontal and anterior cingulate cortex, and several nuclei of the thalamus. Indeed normal neural structure in morphometric imaging may not guarantee normal function.

Bipolar disorder

Studies of physiopathology in BP have identified brain structural/functional and connectivity alterations associated with the prominent mood swings -*i.e.,* alternating recurrent episodes of mania and depression with psychotic symptoms in some cases. Neuroimaging has convincingly showed that bipolar disorder (BP) is a brain disease involving multiple abnormal brain structures -AmyG, HipF, PFC, thalamus and basal ganglia- and neuronal circuits, particularly the limbic-AmyG-thalamic-cortical pathways interconnected by excitatory glutaminergic projections and the limbic-cortical-striatal-pallidal-thalamic circuits[71]. These two networks share components and regulate AmyG response in complex emotions such as melancholic feeling and neuroendocrine/diurnal rhythms. Functional imaging, which permits direct examination of functional brain structures, find decreased blood flow and metabolism in PFC during depression and a reverse increased metabolism during mania[72]. Additionally, postmortem histopathology shows reductions in cortex volume, glial cell counts, and neuronal size in PFC, AmyG, basal ganglia and dorsal raphe nuclei[73,74]. The reduction of glial cells oligodendroglia and microglia that a play critical role in modulating neurotransmission- provides new insights into possible key CNS cellular abnormalities in BP. Altogether, the altered brain structure/network characteristics suggesting that BP is a developmental disease and the identical-twin concordance rates/adoption studies/family history confirm that it has a strong genetic component. The involvement of multiple genes and their epigenetic (psychosocial and environmental interaction)/epistasis (genes interaction) effects make the clear-cut elucidation of altered risk gene expression particularly challenging, although recently the Human Genome Project has helped to overcome some of these difficulties. Linkage and GWAS have reported many BP risk genes, including *ANK3*, *ZNF804A* (important for white matter integrity)[75], *NCAN* (cortical thickness)[76], *TCF4* (ventricular volume)[77], *CACNA1C* (functional connectivity during executive task. *CACNA1C* encodes a subunit of the L-type voltage dependent calcium channel[78] that seems to be involved in the dendritic arborization activity of CRMP3 in HIP)[38], *BDNF* (executive function deficit, neurotrophic factor, plasticity)[79]. Importantly, meta-analyses of the polygenic score profile indicate a large molecular overlap in vulnerability alleles for BP and schizophrenia. In BP, CRMP2 protein levels are decreased in the CA2/CA3 areas and the frontal cortex whereas CRMP4 is decreased in HIP[80].

MDD

MDD is often a recurrent and severe psychiatric disease characterized by decreased density in dendrites and dendritic spines in hippocampus that can be reversed by antidepressant treatments[81]. In the past, most studies have focused on mono-amine system. Another etiological hypothesis proposes that deficiency of neurotrophic factors may mediate depressive symptoms. BDNF is one such factor and there are numerous reports of reduced BDNF in MDD[82]. It has been suggested, in a gene-environment interaction network analysis, that BDNF polymorphism may be involved in MDD. Other evidence which strengthens this hypothesis is provided by studies in BDNF mutant mice[83]. However there are enormous gaps in our understanding of MDD and looking beyond mono-amine and neurotrophic mechanisms to explore the complex neuronal network topologies influence[84] may bring new effective treatment. This notion has received considerable experimental support: (1) it has been shown that the level of CRMP2 important for growth cones formation and neurite arborization is decreased in the brain of patients with depression[85]; (2) recent neuroimaging studies highlight structural alterations in various brain areas of MDD patients[86] -predominately in the HIP and PFC, suggesting overlapping brain abnormalities between the main mental disorders- while the resting-state functional magnetic resonance imaging provides evidence of major change in HIP-PFC circuits[87] responsible for action responses, emotion, sleep, EEG synchronization, attention and memory; (3) the structural/functional abnormalities may contribute to disturbance in mood and cognition in MDD patients and strengthen the hypothesis that MDD is associated with the breakdown of the healthy neuronal networks circuitries; and (4) animal models of depression present similar neuronal dystrophy, reduced synaptic density in PFC and pyramidal cells of the HIP[83].

Post-traumatic stress disorders

After exposure to a traumatic event, *e.g.,* war-related events, physical assault, violence, a small percentage of individuals develop post-traumatic stress disorders (PTSD), characterized by re-experiencing the event with emotional numbing and hyper-arousal symptoms. The identification of structural brain abnormalities, biological and genetic risk in PTSD is required to identify the causal pathways, and inform treatment. Neuroimaging studies of PTSD patients reveal structural and functional alterations within HIP, AmyG, medial frontal cortex and bilateral orbito-frontal cortex[88]. The functional

connectivity studies show that PTSD patients exhibit diminished levels of connectivity between the posterior cingulated cortex region and the right superior frontal cortex and the left thalamus during the resting state[89]. Other biological findings detect the dysregulation of HPA axis and release of corticosteroids -critically involved in mediating the deleterious effect of stress including the decrease of dendritic spines density in HIP in line with the decrease of BDNF[90].

Neuropathic pain

Neuropathic pain (NP)[91] is linked either to peripheral nervous system lesions with drastic changes in gene expression pattern, protein interaction network and non-coding RNAs (*i.e.,* likely induced by inflammatory molecules such as histamine, prostaglandins or bradikinin)[92,93] or in relay structures of CNS (*i.e.,* arisen from metabolic disorders, traumatic injury or neurotoxicity)[94]. These changes can persist long after the initial injury (nerve loss, phantom limb). Common causes of NP are acute or chronic trauma, neurotoxins, diabetes, tumor compression, viral infections or side effect of chemotherapy. A systematic approach of NP is based on the characterization of all pain aspects, including emotional, behavioral, psycho-social, anatomical, genetic and molecular genetic factors[95,96]. Neuroimaging studies provide evidence suggesting that NP is associated with structural, functional and neurochemical alterations distributed across multiple brain structures and networks[97]. However, because many environmental factors may interact with genetic polymorphism to influence pain perception, data from proteomic studies to elucidate genetic contribution to NP remain limited and inconsistent although diverse molecules (NMDA/AMPA/ P2X3 ion channel receptors, G-protein-coupled receptors, AnnexinV/CaM/CRMP2 calcium signaling protein, N-type voltage-gated calcium channels CaV2.2, receptor tyrosine kinases, trkB for BDNF, non-coding RNAs)[98-101] can account for changes that arise in pathological pain states.

TREATMENT OF NEURAL NETWORK DEFICITS AND NP

Remodeling neuronal connectivity by transcranial magnetic stimulation

Transcranial magnetic stimulation (TMS) consists of promoting a localized electric current through a localized magnetic field produced by a TMS-coil[102]. It is assumed that TMS and repetitive TMS have an inhibitory or facilitatory effect on neurons and neuronal networks. They can induce plasticity, modulate neurotransmission and increase neuroprotection against oxidative effect via BNDF/TrkB signaling system in the stimulated site and in other structures functionally connected with it[103-105]. These effects associated with magnetic stimulation -low intensity stimulation results in neurite sprouting and increase in synaptic contacts while high intensity stimulation has devastating effects- can be maintained as long as 6 mo after treatment[106]. Furthermore, it has been shown that TMS reduces structural, functional and behavioral abnormalities in ephrin-A2A5$^{-/-}$ mice but do not adversely affect the control WT[107]. Altogether, these observations suggest that TMS treatment may modulate synaptic strength not only locally but at distant sites, modulating the connectivity networks and offering the hope of a focal intervention capable of ameliorating the altered circuitries underlying psychiatric disorders. Indeed clinical trials directed to the treatment of major depression, schizophrenia, bipolar disorders, anxiety disorders, PTSD and neuropathic pain have supported a possible therapeutic effect of TMS alone or in combination with drugs[108]. Although the mechanisms of TMS activity are hypothesized to be based on induction of neuronal firing, certain effects may be due to the cryptochrome (CRY)/photolyase family: Present in all cell nuclei, CRYs that contain magnetosensitive radical pairs may provide the abilities of cells to specifically respond to magnetic field[109,110].

Remodeling neuronal connectivity with selective modulation of BDNF/CRMPs expression

One of the most studied and best characterized neurotrophins in CNS is BDNF. It has received remarkable attention from scientists because it is essential for neurogenesis, neuronal differentiation (including neuritogenesis/dendritic arborization, spine formation and axonogenesis *via* nonphosphorylated CRMP2), survival, migration, apoptosis, synaptic plasticity (*via* TrkB and p75NTR activation) and neuronal network formation, and from clinicians because it is required for normal development/functions of the brain and its expression is found decreased in several brain regions in post-mortem studies of patients with neurodegenerative and psychiatric diseases[60,79,81-83,111,112]. In physiological condition, BDNF activity depends on the activation of its downstream intracellular signaling cascades -Ras/MAPK, PLC-γ, PI3K/AKT. An additional level of regulation is provided by the balance between neurotrophic signaling through mature-BDNF/TrkB and apoptotic signaling through pro-BDNF/p75, which determines which connections are maintained within the neuronal network and which neurons are eliminated. Importantly, altered BDNF activity has been reported in brain pathology, particularly in limbic structure, AmyG, orbital and medial PFC and related keys circuits. Antidepressant activity seems to be linked to increasing levels of BDNF. Similar to BDNF, CRMPs express abundantly in the nervous system and while manipulations of their expression by RNAi, gene targeting or overexpression has confirmed their critical role in axono-dendritic growth and collapse, neuronal migration/survival and spinogenesis[29,30,33,34,36], other studies reveal that their expression is also altered in human pathologies including mental disorders[33,85,113-115]. Restoration of altered BDNF/CRMPs activities in these affected areas and/or affective circuits with newer and more refined/targeted immuno-

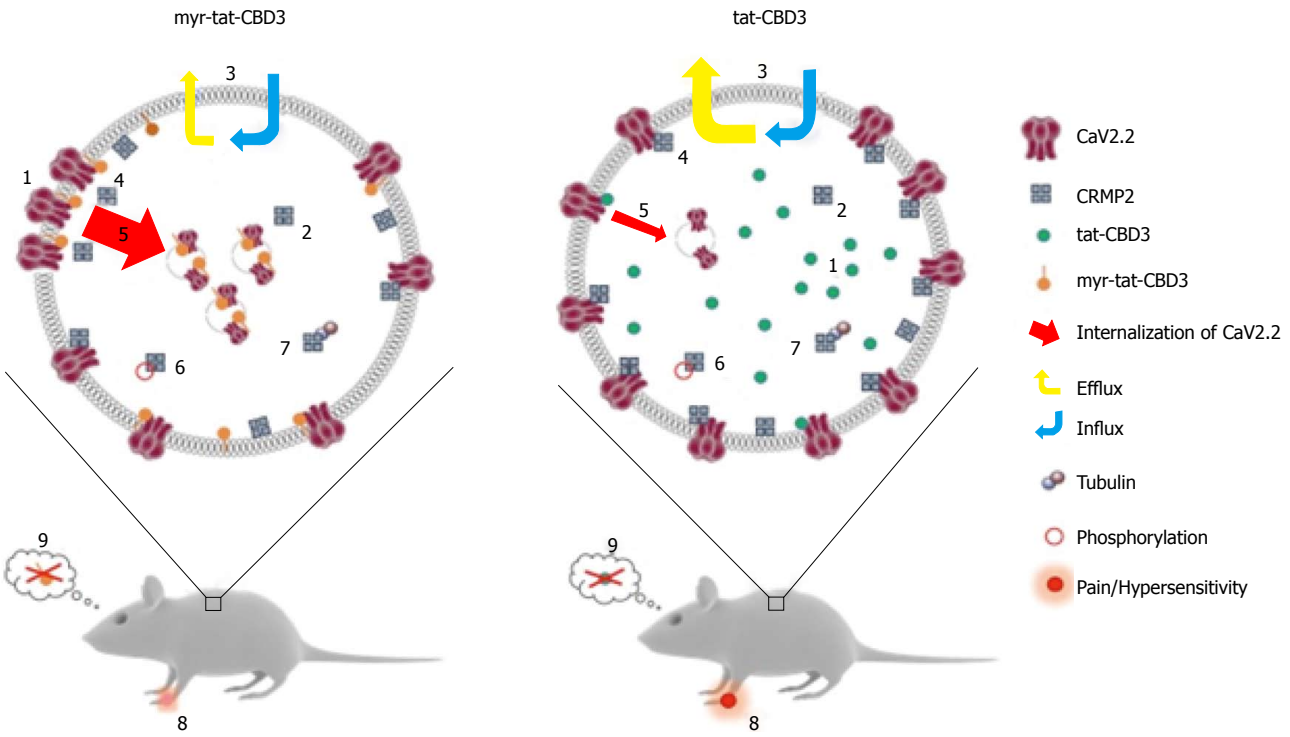

Figure 3 A possible model for N-myristoylated collapsin response mediator protein 2 peptide's actions on CaV2.2 trafficking and efficacy in neuropathic pain model. Application of an N-myristoylated tat-conjugated CRMP2 peptide (myr-tat-CBD3) results in membrane-delimited "rimming" of the peptide whereas the non-myristoylated version (tat-CBD3) appears to be spatially diffusely distributed in the cell cytoplasm. Analysis of penetration of peptides into GPMVs, which are "blebs" of membrane devoid of organelles and actin cytoskeleton, reveals an unrestricted distribution of the membrane sensitive dye (di-4-ANNEPDHQ) with tat-CBD3, whereas the myristoylated peptide induces a lateral heterogeneity of the fluorescent signal resulting in dye aggregation into micro domains within these model membranes[122]. While CRMP2 has been demonstrated to exist as a tetramer, the oligomeric state of membrane proximal CRMP2 is as yet unknown; however, neither peptide appears to affect CRMP2 oligomerization[123]. Whereas the cells take up both forms of the peptide with similar efficiency, the myristoylated peptide demonstrates a lesser degree of efflux[124]. The apparent increase in retention of myr-tat-CBD3 translates into a superior potency and efficacy in inhibition of evoked calcium influx in sensory neurons presumably *via* greater uncoupling of CRMP2-CaV2.2 interactions at or juxta-membrane[125]. Increased inhibition of CaV2.2 surface trafficking induced by myr-tat-CBD3 compared with tat-CBD3 may account[126] for the more pronounced restriction of calcium influx imposed by the myristoylated peptide. Cdk5-phosphorylated CRMP2 has been demonstrated to have an enhanced interaction with CaV2.2[129]; however myr-tat-CBD3 does not affect the levels of Cdk5-phosphorylated CRMP2[127], thereby ruling out a role of phosphorylated CRMP2 in regulating calcium influx. CRMP2 binding to tubulin is strengthened by the peptides[130]; the consequences of this are currently unknown. Importantly, where tat-CBD3 is completely ineffective in reversing mechanical hypersensitivity in a rat neuropathic pain model (tibial nerve injury), the myristoylated peptide reverses this hypersensitivity when administered *in vivo*[122]. Neither peptide elicits any reward-like addictive behaviors. GPMVs: Giant plasma membrane vesicles; CRMP: Collapsin response mediator proteins.

pharmacological agents will likely yield more effective treatments, particularly in treatment-refractory cases, and greater understanding of the mechanisms underlying mental disorders.

NP treatment with CBD3, a peptide derived from CRMP2

Presently, the understanding of the molecular and cellular mechanisms of NP is incomplete, and new concepts are needed to improve its treatment. Genetic and clinical studies have validated N-type voltage-gated calcium channels (CaV2.2) as targets for NP treatment[116-118] but selective blockers have potential serious side effects. Targeting protein interactions to direct channel block has been proposed[119]. This approach has yielded a novel peptide-derived therapeutic prototype for NP relief: A CRMP2-derived peptide (tat-CBD3; Figure 3) that disrupts the CaV2.2/CRMP2 interaction shows anti-nociceptive activity in animal models of neuropathic pain[120]. The relative lack of toxicity provides evidence that CBD3 has therapeutic promise[121]. Recent efforts to optimize the peptide's efficacy resulted in the generation of a myristoylated version of the peptide; myr-tat-CBD3 -a myristoylated CBD3 peptide harboring a 14-carbon fatty acid, myristate, onto an N-terminal glycine[122]. *N*-myristoylation is a lipid anchor modification of eukaryotic and viral proteins targeting them irreversibly to locate to the membrane[123-125]. Tethering the CBD3 peptide to the membrane through myristoylation confines its action(s) to the uncoupling of membrane CaV2.2-CRMP2 and blocks Ca^{2+} influx without affecting the CRMP2 functions mediated by interactions with other cytoplasmic proteins[126].

As summarized in Figure 3, the myr-tat-CBD3 peptide is equal or better in its efficacy to tat-CBD3 on reducing pain-related behaviors with pronounced reversal of mechanical hypersensitivity in a postoperative incisional pain model[127] at dose of 0.1 mg/kg in contrast to the lack of effect observed even at 20 mg/kg of tat-CBD3[122]. Moreover sustained relief (> 6 wk) of NP was obtained with an AAV-targeted expression of CBD3 peptide in rat DRG[128]. Both tat-CBD3 and myr-tat-CB3 reduce pain-like behaviors without demonstrating any

reward-like potential. These experiments demonstrate the possibility of tailoring molecules that affect membrane targets for specific inhibition of CaV2.2-CRMP2 interactions (Figure 3).

CONCLUSION

Data from various basic science studies together with experimental/clinical studies suggest a correlative causal link between specific neural networks dysfunctions and mental disorders. However, one could argue that (1) a correlation between experimental and clinical observations is a correlative statement but not a causal demonstration; (2) an observed electrical current dysfunction may not support this common view of correlative network specificity because it can result from different network stimulations with many unknown parameters and consequently underspecifying any structural function; and (3) more direct experiments will be needed to consolidate these observations. This approach is critical since it may raise further technical inventions/interventions and challenges such as generating new tools more powerful optogenetic/acousto-optical deflectors approaches in animal models[131,132] to visualize, control and compute simultaneously single neuronal activity and neuronal circuitries, to integrate in parallel behavioral measures for a better treatment of mental diseases, less side-effects associated to newer psychotropic drugs and gene therapy[133-137], and more importantly a better understanding of the physiology and pathology of the human brain in healthy activity and abnormal behavior respectively.

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REVIEW

Role of astrocytic glutamate transporter in alcohol use disorder

Jennifer R Ayers-Ringler, Yun-Fang Jia, Yan-Yan Qiu, Doo-Sup Choi

Jennifer R Ayers-Ringler, Neurobiology of Disease PhD Program, Mayo Graduate School, Mayo Clinic, Rochester, MN 55905, United States

Yun-Fang Jia, Yan-Yan Qiu, Doo-Sup Choi, Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic College of Medicine, Rochester, MN 55905, United States

Author contributions: All authors contributed equally to conception and design of the manuscript, analysis and interpretation of data, drafting, and making critical revisions of the manuscript; all authors gave final approval of the version of the article to be published.

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Correspondence to: Doo-Sup Choi, PhD, Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic College of Medicine, 200 First Street SW, Rochester, MN 55905, United States. choids@mayo.edu
Telephone: +1-507-2845602
Fax: +1-507-2841767

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Abstract

Alcohol use disorder (AUD) is one of the most widespread neuropsychiatric conditions, having a significant health and socioeconomic impact. According to the 2014 World Health Organization global status report on alcohol and health, the harmful use of alcohol is responsible for 5.9% of all deaths worldwide. Additionally, 5.1% of the global burden of disease and injury is ascribed to alcohol (measured in disability adjusted life years, or disability adjusted life years). Although the neurobiological basis of AUD is highly complex, the corticostriatal circuit contributes significantly to the development of addictive behaviors. In-depth investigation into the changes of the neurotransmitters in this circuit, dopamine, gamma-aminobutyricacid, and glutamate, and their corresponding neuronal receptors in AUD and other addictions enable us to understand the molecular basis of AUD. However, these discoveries have also revealed a dearth of knowledge regarding contributions from non-neuronal sources. Astrocytes, though intimately involved in synaptic function, had until recently been noticeably overlooked in their potential role in AUD. One major function of the astrocyte is protecting neurons from excitotoxicity by removing glutamate from the synapse *via* excitatory amino acid transporter type 2. The importance of this key transporter in addiction, as well as ethanol withdrawal, has recently become evident, though its regulation is still under investigation. Historically, pharmacotherapy for AUD has been focused on altering the activity of neuronal glutamate receptors. However, recent clinical evidence has supported the animal-based findings, showing that regulating glutamate homeostasis contributes to successful management of recovery from AUD.

Key words: Alcohol; Addiction; Glutamate; Astrocyte; Excitatory amino acid transporter type 2; Glia; Striatum

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Core tip: Review of astroglial involvement in alcohol use disorder and potential for astrocyte-specific glutamate transporter excitatory amino acid transporter type 2 (EAAT2, Slc1a2)/GLT-1 in pharmacological treatment, including alcohol withdrawal symptoms.

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INTRODUCTION

Substance abuse and addiction is a pervasive problem at all socioeconomic levels worldwide. Alcohol, though one of the most common addictive substances, is recreationally used by many people without incident. Alcohol use disorder (AUD) or alcohol addiction often results in one of the most lethal sets of withdrawal symptoms, including seizures, hallucinations, and delirium tremens, termed alcohol withdrawal syndrome (AWS). As with most addictive substances, craving and relapse rates are high, and predictability, and even recognition, of addiction is difficult to ascertain. This makes alleviation of withdrawal symptoms the primary focus on treating AUD.

Like other neuropsychiatric conditions, AUD involves dysfunction of normal signaling pathways. Importantly, neurons are not the only cells involved in neural signaling. The majority of cells in the central nervous system (CNS) are of the glial lineage[1]. Two of the major glial cell types, oligodendrocytes and microglia, have been well characterized, as they are relatively monofunctional: Oligodendrocytes myelinate neurons, and microglia regulate the CNS immune response[2]. Ironically, the most abundant glia, the astrocyte, is not as well understood. Its diverse role in maintaining vascular integrity and metabolic homeostasis, and regulating synaptogenesis and immune response[1,3], means that its morphology and gene expression vary greatly depending on its location, local contacts, and microenvironment[4,5].

AWS is attributed to elevated extracellular glutamate levels in certain brain regions, such as the striatum and hippocampus[6-8]. These regions are part of the mesocorticolimbic circuit, which is highly implicated in addiction disorders[9,10]. Astrocytes provide critical regulation of synaptic glutamate concentrations in these regions through bi- and uni-directional transporters. Glutamate, the most important excitatory neurotransmitter in the CNS, serves to stimulate action potentials by activating its cognate ionotropic receptors on the postsynaptic neurons, and modulate synaptic connectivity through its cognate metabotropic receptors. Astrocytes are responsible for removing glutamate from the extracellular space, thereby limiting its effects. Within the astrocyte, glutamate is converted to glutamine *via* glutamine synthetase, and released back out to the extracellular environment for uptake by presynaptic neurons, which convert it back to glutamate to be packaged into vesicles for synaptic release. In AWS, this glutamatergic tone is dysregulated, resulting in a hyperglutamatergic state that can lead to neuronal excitotoxicity. Clinically, benzodiazepines are often used to alleviate withdrawal symptoms, but patients can become physically dependent on these, as well[11].

Animal models have been extremely useful in studying addiction. The rodent corticolimbic circuitry is very similar to human, and thus provides an effective method for studying various aspects of the addiction and withdrawal processes. Certain species are useful for studying behaviors of different points and aspects of the addiction process, including consumption, preference, learning, tolerance, and withdrawal[12]. Additionally, through genetic manipulation of specific components of glutamatergic signaling, we have come to understand ethanol's effects on involved neuronal glutamate receptors, such as NMDA and AMPA receptors[13-17]. This review will focus on the role of glia in neuronal function, both normal and pathological, and illuminate the importance of understanding astrocyte function in neuropsychiatric disorders, like AUD.

GLIAL FUNCTION IN NEUROPSYCHIATRIC DISORDERS

Glial cells are mostly known for their contributions to physiological processes and pathological involvement. There are many more glial cells than neurons (glia to neuron ratio = 4:1), and they are generally smaller[18]. Three subtypes have been identified in the CNS: Astrocytes, microglia and oligodendrocytes. The major function of glial cells is to maintain homeostasis in the CNS, including supporting blood vessels, providing nutrition and oxygen to neurons, regulating neurotransmitter metabolism and modulating the immune response. Therefore, balance disruption derived from any of these cell types could be associated with multiple kinds of psychiatric disorders.

Oligodendrocytes are one of the more numerous than the types of glial cells. Their primary role is to myelinate axons, which increases the resistance and reduces the effective capacitance of axonal membranes, accelerating the transmission of electrical signals. They arise from a large population of oligodendrocyte precursor cells (OPCs), a source of replacement cells for damaged oligodendrocytes after injury or in disease. There are multiple trophic and growth factors that maintain oligodendrocyte survival and myelination[19]. One therapeutic option is to increase and amplify this OPC pool once the pathologically activated signa-

ling pathways are identified[20]. Moreover, mounting evidence shows that oligodendrocytes may also play a critical role in neuropsychiatric disorders[21].

Astrocytes represent highly heterogeneous mediators that interact directly with neurons, which is crucial for maintaining brain function. Astrocytes play an essential role in neurogenesis and the development of the CNS, energy metabolism, synaptic signaling, immune defense, amino acid neurotransmitter clearance, neurotrophin production and ionic homeostasis[22]. More recently, many studies have suggested that astrocyte dysregulation is associated with neuropsychiatric disorders, such as Wernicke's encephalopathy[23] and Korsakoff psychosis[24], schizophrenia[25], depression and addiction[26].

Microglia originates from hematopoietic progenitors and have been considered the resident immune cells in CNS. However, recent evidence has provided a notion that microglia are not only activated by inflammatory challenge or neuronal damage, but that they extend an array of physiological functions through synaptic maturation[27], maintenance of CNS homeostasis, memory processes and neurogenesis[28]. Additionally, disturbance of microglia responding to minor pathological intrusion is implicated in multiple neuropathological and neuropsychiatric disorders.

Pathological potential of neuroglia in AUD

Astrocytes play critical roles in CNS abnormalities associated with ethanol-induced neurotoxicity. Chronic ethanol exposure profoundly affects the expression of GFAP, an astroglial stress fiber, as well as the function of astrocytes. One interesting study found that by increasing astrocyte number by injecting purified astrocytes into rat forebrain learning and memory abilities associated with cholinergic signal recovery were improved[29]. It is well known that alcohol and its metabolite acetaldehyde show direct neurotoxicity by disturbing thiamine-related enzymes, resulting in thiamine deficiency and damage to astrocytes[30]. Our laboratory demonstrated the importance of adenosine signaling in regulating alcohol-related behaviors. Mice lacking type 1 equilibrative nucleoside transporter (ENT1), the glial transporter responsible for maintaining adenosine tone in the synapse, consume more ethanol in two bottle choice experiments when compared to wild-type littermates[31]. In addition, antagonists and agonists acting on the adenosine A_{2A} receptor ($A_{2A}R$) modulate ethanol preference and withdrawal symptoms. Moreover, inhibition of $A_{2A}R$ in the dorsomedial striatum promoted goal-directed behavior and increased sucrose or ethanol drinking[32]. We revealed that deletion of ENT1 decreased the expression of astrocytic glutamate transporter GLT-1, the protein responsible for regulating extracellular glutamate concentration in the striatum. Adenosine-mediated glutamate signaling in neuroglial interaction in ethanol intoxication and preference has been thoroughly described elsewhere[33,34].

Microglia are the resident macrophages and are distributed ubiquitously throughout the CNS. Alterations in microglia have even been noted in AUD models. After intensive chronic ethanol intoxication in rats, there were structural lesions in the CNS, including infiltration of mononuclear cells and lymphocytic-microglial nodules[35]. Changes in immune-related genes have been found in human alcoholic brain, including chemokines (Ccl2, Ccl3) and chemokine receptors (Ccr5, Ccr1 motif)[36]. Consumption and preference of ethanol decreased in mice after deleting either chemokines (Ccl2 or Ccl3) or chemokine receptors (Ccr2). Particularly the proinflammatory cytokine, CCL2, was extensively higher in alcoholics[37]. Also, consecutive 10-d injection of ethanol increased pro-inflammatory cytokine TNFα in the brain. Meanwhile, ethanol enhanced LPS-induced increases in TNFα, MCP-1, and IL-1β in brain[38]. Previous studies indicated that TLR4 response might be a vital mechanism of chronic ethanol-induced neuroinflammation. The signaling pathways related to IL-1RI/TLR4 receptors were facilitated by chronic ethanol treatment[39], while the deletion of TLR4 prevented ethanol-induced glial activation and production of inflammatory mediators[40].

Regarding oligodendrocytes, alcohol appears to have different effects, but much of the evidence points toward reduced oligodendrocyte function. Prenatal alcohol exposure decreased the level of myelin basic protein (MBP) expression[41], consequently leading to a reduction in brain myelination that may contribute to the development of fetal alcohol syndrome[42]. Protein kinase C was activated after ethanol exposure, which delays MBP expression in differentiating CG-4 oligodendrocytes. Oligodendrocyte myelin glycoprotein was significantly decreased in rat hippocampus by chronic ethanol exposure[43]. This deficit may explain the demyelination observed in human alcoholics[44]. Recent studies showed that premyelinate oligodendrocytes and myelin-associated protein were decreased in medial prefrontal cortex (mPFC) during chronic intermittent ethanol vapor exposure (CIE). However, protracted abstinence increased MBP levels in the mPFC, possibly a compensatory effect after CIE[45]. In contrast, the activity of enzyme 2′, 3′-cyclic nucleotide 3′-phosphodiesterase, a maker for oligodendrocytes, was increased in ethanol-treated cultures[46].

Neuroglia pathology in other psychiatric conditions

Glial pathology is also implicated in other neuropsychiatric conditions, like schizophrenia and bipolar disorder (BD). One study demonstrated decreased astrocytic gene expression in the deep layers of the anterior cingulate gyrus, including excitatory amino acid transporter 2 (EAAT2)[47]. In this regard, animal models have been somewhat helpful in investigating structural changes, glycogen metabolism and glutamate signaling[48].

BD, like AUD, involves the corticostriatal circuit. Morphometric postmortem and neuroimaging studies found decreased glial cell density in the mPFC of

patients with BD[49]. Recent studies indicate that chronic treatment with anti-bipolar drugs could inhibit astroglial glutamate release[50]. Additionally, S100β, a neurotrophic factor mainly released by astrocytes, was increased after chronic lithium treatment, although astrocytes or interaction between neurons and astrocytes were unaffected[51].

Regarding microglia, studies in schizophrenia patients have yielded more consistent results. Microglia are activated along with increased cell population in the postmortem brains of schizophrenic patients[52]. Consistently, the level of cytokines, IL-1β, IL-6, and TGF-β, were elevated in schizophrenia. Interestingly, these alterations were reversed by antipsychotic treatment[53]. The dysfunction of microglia is associated with impairment of nervous system development, synaptic formation and pruning[27,54], processes that may also affect neural circuitry in AUD.

Neuroinflammation has also been linked to BD. The level of IL-1β was increased in cerebrospinal fluid of euthymic BD patients when compared with normal controls, and even higher in those who had recently experienced one or more manic/hypomanic episodes[55]. In addition, microglia activation induced higher levels of IL-1β, which was associated with increased suicide behavior in BD patients[56]. Moreover, concentrations of pro-inflammatory cytokines, which are associated with increased activation of MAPK and NF-κB pathways in BD patients, were higher, as well. These results suggest that microglia play a potentially crucial role in BD. Interestingly, there is an increased incidence of BD in patients with the autoimmune disorder, multiple sclerosis, where oligodendrocytes are targeted by the immune system. And, oligodendrocytic markers are down regulated in patients with BD[57].

Multiple studies have confirmed oligodendrocytes and myelination are impaired in schizophrenia[58-60]. Oligodendrocytic and myelination abnormalities damage saltatory conduction and information transfer between neurons in schizophrenia[61]. White matter anomalies were found in schizophrenia patients from maternal and early postnatal immune challenge[62], perinatal hypoxia[63], and during childhood and adolescence[58,64], suggesting that damage to axonal integrity and conduction velocity may play a causal role in schizophrenia.

Taken together, studying glial cells is warranted for understanding neuropsychiatric disorders in regards to glutamatergic abnormalities.

SYNAPTIC, PERISYNAPTIC AND EXTRASYNAPTIC ASTROCYTIC PROCESSES IN REGULATING GLUTAMATE SIGNALING

Neurons connect to each other with a kind of chemical junction called a synapse, which consists of the presynapse, post-synapse, and synaptic cleft as a key information communication relay and central functional element of the nervous system[65,66]. Each neuron can receive a number of synaptic inputs and these synapses have diverse properties, including the type of neurotransmitter and the number of postsynaptic receptors. Neurotransmitter vesicles accumulate in the presynaptic terminal, while the neurotransmitter receptors located in the postsynaptic neurons are recruited to the postsynaptic.

Synaptic astrocytic processes

Astrocytes were widely thought to provide only metabolic and physical support for neurons, but now they are demonstrated to directly participate in neuronal signaling, even locally at synapses[67,68]. Many investigations have shown that astrocytes exhibit unique biophysical and functional electrical properties, are sensitive to neuronal activity and are actually involved in the control of synaptic transmission. Astrocytes are indeed equipped to sense and integrate neuronal information through ionic channels, neurotransmitter receptors and transporters, and intracellular signaling pathways[69]. Astrocytes are also known to influence synaptic activity *via* synthesizing and recycling glutamate[70] and responding to synaptic release of neurotransmitters[68,71,72]. Glutamate, as a major neurotransmitter in the brain, exerts a critical role in mediating synaptic activity and also causing a response in astrocytes[73,74]. Importantly, the involvement of astrocytes in glutamatergic regulation is very widespread, as most glutamate is taken up by transporters expressed on astrocytic membranes when it is released into the extracellular space between neurons[75,76], and it is estimated that only 20% of synaptic glutamate is taken up by transporters on the postsynaptic neurons[77]. Astrocytes can synchronize with neuronal activity, and subsequently regulate glutamate transmission between neurons[78]. Astrocyte-to-neuron glutamate signaling may mediate activity-dependent modulation of inhibitory synapses in the hippocampus, with glial glutamate release taking place at some distance from the synapse[79]. The increases in calcium (Ca^{2+}) evoked by neuronal activity results in a release of glutamate from astrocytes[80,81]. This release of glutamate process is known to be a Ca^{2+}-dependent exocytic pathway[82]. In addition, during synaptic activity neurons or astrocytes release ATP, which cause an increase in intracellular (Ca^{2+}), mediated by the P2Y1 type purinergic receptors (P2Y1Rs) in hippocampal astrocytes[83]. As a G protein coupled receptor, P2Y1R signaling in astrocytes is coupled to Ca^{2+}-dependent glutamate exocytosis[84,85].

Glutamate is able to induce a wide range of effects in astrocytes *via* metabotropic glutamate receptors (mGluR), NMDA receptors, and AMPA receptors[86]. Hippocampal astrocytes express functional AMPA receptors[87], the properties of which can be changed by astrocytes during postnatal development. It has been reported that immature astrocytes had a prolonged activation of the AMPA receptors, while glutamate

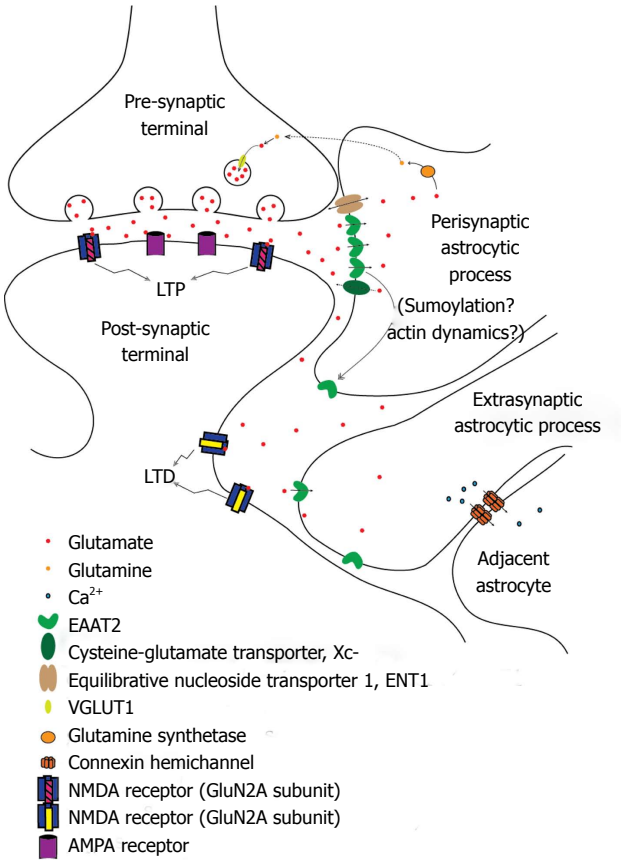

Figure 1 Fine regulation of glutamate levels and excitatory amino acid transporter 2 localization in neuron-glial interaction. The perisynaptic astrocytic process contains a higher concentration of EAAT2 immediately adjacent to the synapse. The EAP also contains EAAT2, but in lower concentrations. Glutamate spillover results in activation of GluN2B-containing NMDA receptors (increasing LTD response in the post-synaptic neuron), increased EAAT2 surface expression on and migration of EAPs, and activation of adjacent astrocytes via calcium waves propagated through connexin channels. LTP: Long term potentiation; LTD: Long term depression; EAAT2: Excitatory amino acid transporter 2; Xc-: Cysteine-glutamate transporter; ENT1: Equilibrative nucleoside transporter 1; VGLUT1: Vesicular glutamate transporter 1; NMDA: N-Nitrosodimethylamine; AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; EAP: Extrasynaptic astrocytic process.

responses were greatly increased as astrocytes matured. In addition, the presynaptic NMDA receptors are activated and the excitatory communication between neurons is increased once astrocytic glutamate is released[85]. Additionally, astrocytes also use the mGluRs to modulate neuronal activity. However, previous results suggest developmental changes in the expression of mGluRs in astrocytes[88], predicting its important role in development. Reports reveal that synaptic glutamate stimulates astrocytic mGluRs and subsequently triggers glutamate release from astrocytes[80,89].

Perisynaptic astrocytic processes

It has recently been recognized that astrocytes act as a third partner in synaptic processes, leading to the description of a "tripartite synapse[90]", with the astrocytic processes in close apposition to the synaptic cleft[91]. Perisynaptic astrocytic processes (PAPs), assumed to mediate this communication, may detect glutamate spillover and other substances from active synapses[76,92]. Glutamate can also cause structural alterations of astrocytes, driving them to extend and modify their own processes[73,93] (Figure 1). It is thought that PAPs position next to the synaptic cleft, as the effectors of glia-neuronal crosstalk at the synapse, to exert their roles in ion buffering and transmitter uptake, thus keeping a low diffusion distance[94]. Glutamine synthetase is mainly expressed in the PAPs around identified glutamatergic synapses[95].

There have been a few approaches that described peripheral astrocyte processes related to synapses using direct or indirect observations. Glial gamma-aminobutyricacid (GABA) receptor channels could be of functional importance in buffering extracellular Cl^- in the perisynaptic microenvironment using patch-clamp techniques, and might relate to mechanisms not present in the peripheral processes[96]. Electrically stimulated parallel fibers increased intracellular calcium concentration in peripheral processes of Bergmann glia, astrocytic cells located in the cerebellum[97]. The transmitter release is affected by reduction in glutamate clearance through modulation of presynaptic mGluR. Therefore, astrocytic wrapping of neurons may contribute to the regulation of synaptic efficacy in the CNS[98]. Glia-synaptic interactions can be attributed to the astrocytic peripheral processes in the perisynaptic microenvironment. As the functions of PAPs displayed might not be representative of the astrocyte as a whole, they could be recognized as a specialized astroglial compartment[94].

Extrasynaptic astrocytic processes

In some instances, glutamate may escape from the synaptic cleft, generating extrasynaptic glutamate dynamics[99,100]. The kinetic simulations of extrasynaptic glutamate uptake show that glutamate can bind to several subtypes of receptors, but transporters rapidly reduce the free concentration of transmitter[101]. Extrasynaptic glutamate dynamics is believed to regulate a variety of important neural and glial functions including synaptic transmission[102], synaptic plasticity[103], synaptic crosstalk[104,105], nonsynaptic neurotransmission[106], neuronal survival[107], and gliotransmitter release[108].

Synaptic "spillover", or extracellular diffusion of transmitters and modulators after extrasynaptic release in the local circuit regions, is an important short distance form of volume neurotransmission. The neurotransmitters bind to extrasynaptic neuronal and glial receptors and then activate related signaling pathways in the neuroglial networks of the brain (Figure 1). G protein-coupled receptor heteromers play a major role in the integrative processes of extrasynaptic signaling on glutamatergic synapses. The balance of activity in the different types of projection neurons is determined by the diverse distributions of high affinity receptors on the neurons and glia, where both neuronal extrasynaptic and long distance volume transmission signaling can be involved[109]. Temporal resolution at axon terminals of

fast-spiking neurons might be due to rapid glutamate receptor desensitization. Transmitter diffusion is partly responsible for transmitter clearance from the synaptic cleft[76]. The glutamate transient duration seen at a distance from the release site can be greatly increased if interactions between glutamate and extrasynaptic binding sites reduce the effective diffusion constant, resulting in a greater probability of NMDA receptor activation[110]. Relatively small amounts of glutamate are likely to activate extrasynaptic receptors, especially if the diffusion coefficient in the extracellular medium is low[101]. Extrasynaptic glutamate spillover implies significant cross-talk between neighboring synapses, which could have major repercussions for information processing[111].

GLUTAMATE TRANSPORTERS IN AUD

Glutamate is arguably the most important neuroexcitatory amino acid in the CNS. As such, its concentration, both intra- and extra-cellularly, is tightly regulated by membrane transporters. Within neurons, the vesicular glutamate transporters, of which there are three known subtypes[112], pack glutamate from the cytosol into presynaptic vesicles utilizing the proton electrochemical gradient[113]. In glia, the cysteine-glutamate transporter (Xc-) is the major transporter responsible for releasing glutamate into the extracellular space, while a family of sodium-dependent transporters, called excitatory amino acid transporters (EAAT), removes glutamate from the extracellular environment. The function of these latter transporters is critical, as excessive synaptic glutamate can lead to neuronal excitoxicity and/or death. There are five mammalian EAAT subtypes (EAAT1-EAAT5) that have been identified to date. EAAT1 and EAAT2 (in rodents, designated glutamate and aspartate transporter, GLAST, and glutamate transporter 1, GLT-1, respectively) are the primary subtypes responsible for extracellular glutamate clearance throughout the CNS during neurotransmission, and are predominantly expressed in astrocytes[114,115]. EAAT1 is most abundantly expressed in the cerebellum, while EAAT2 has a more ubiquitous expression pattern, which is strongest in the forebrain[116]. EAAT3 (EAAC1 in rodents) is the most widely distributed, expressed in peripheral tissues, as well as in both neurons and astrocytes[117]. EAAT4 is most strongly expressed in cerebellar Purkinje cells[118], and EAAT5 is almost exclusively expressed in the retina[119].

In recent years, EAAT2 has received increasing attention in addiction research, as it is the primary EAAT subtype expressed in the striatum, a component of the basal ganglia that has been implicated in addiction and other psychiatric disorders, and is responsible for clearance of at least 90% of extracellular glutamate. Conceptually, alterations in EAAT2 surface expression should impact neurotransmission, and indeed, this has been demonstrated[120,121].

Structure, function and localization

Structurally, EAATs contain eight transmembrane segments. The N-terminal structures are involved in intersubunit contacts, while the C-terminal half is implicated in substrate transport[122]. EAATs appear to be oligomeric, and EAAT2 tends to form homotrimers and heterotrimers with its other isoforms[123,124], generating a bowl-shaped complex with the aqueous, concave side facing the extracellular space and the pointed side facing the cytoplasm. Interestingly, between the subunits on the hydrophobic transmembrane sides are distinct crevices that allow lipids to interact with key functional domains of the transporter[122], suggesting a role for lipid modulation of EAAT2 activity.

EAATs are capable of transporting both glutamate and aspartate. With one molecule of glutamate, they co-transport three sodium ions and one proton in exchange for one potassium ion[122]. Mouse EAAT2 (GLT-1) shares 97% sequence homology with humans and 99% with rats[125]. The Km of EAAT2 is fairly small (for D-aspartate, 17 ± 5 μmol/L for mouse[126], 54 ± 9 μmol/L for human[127]). Synaptic levels of glutamate can be as high as 1 mmol/L[128], so transport of glutamate out of the synapse occurs very rapidly. In this way, EAAT2 maintains low extracellular glutamate concentrations, preventing it from reaching excitotoxic levels.

In the striatum, EAAT2 is primarily located on the membrane surface of astrocytes, but can also be found on the outer mitochondrial membrane, on rough endoplasmic reticulum, and on polyribosomes[129]. EAAT2 tends to concentrate at cellular processes adjacent to glutamatergic synapses (PAPs; Figure 1) and in astrocytic endfeet near capillaries in the neurovascular unit[1]. It is tightly associated with cholesterol-rich lipid rafts[130,131], and it has been suggested that it may be part of a macromolecular complex that also contains Kir4.1 and AQP4 at capillary endfeet, implying a combined effort for glutamate and potassium ion homeostasis[132].

Glutamate transporter regulation

EAAT2, like most proteins, can be regulated by multiple methods. It is subject to transcriptional regulation by transcription factors and differential splicing, epigenetically by histone modifications and DNA methylation, post-translationally by various residue modifications, and functionally by certain toxins, membrane lipid composition, and cytoskeletal rearrangements[115,131,133-137] (Table 1 provides a more complete list of modulators).

The EAAT2 promoter contains several transcription factor binding sites, including CREB, KBBP, NF-κB, N-myc, Sp1 and Yin Yang 1[115,134,138]. The most studied of these, NF-κB, has been shown to be an effective regulator of EAAT2 expression, though by different mechanisms depending on the initiation factor[139-145]. For example, EGF-receptor stimulation induces increased EAAT2 transcription, but not *via* IκB degradation[115], whereas Ceftriaxone, a β-lactam antibiotic, does appear to

Table 1 Modulators of excitatory amino acid transporter 2 expression and/or function

Compound	Demonstrated effect on EAAT2	Process/pathway	Ref.
17β-estradiol	Increased expression	Activation of both NF-κB and CREB	Lee *et al*[142]
cAMP/bromo-cAMP/dB-cAMP	Increased expression	PKA pathway, PI-3K and NF-κB	Su *et al*[115]
Ceftriaxone	Increased expression	Conventional NF-κB activation	Lee *et al*[146]; Rothestein *et al*[143]
EGF	Increased expression	Non-canonical NF-κB activation	Zelenaia *et al*[144]
PACAP	Increased expression	PKA and PKC activation	Figiel *et al*[139]
Parawixin1	Increased transport speed	Allosteric enhancement of receptor function	Fontana *et al*[133]
Raloxifene	Increased expression	Activation of both NF-κB and CREB	Karki *et al*[135]
Riluzole	Increased expression and activity	Multiple pathways, mediated by estrogen receptors and HSF1	Karki *et al*[135]; Liu *et al*[137]
Tamoxifen	Increased expression	Activation of both NF-κB and CREB	Karki *et al*[141]
TGF-α	Increased expression	Tyrosine Kinase activation	Lee *et al*[136]; Su *et al*[115]
TNFα	Decreased expression	Co-activation of NF-κB and N-myc	Sitcheran *et al*[145]

Listed in this table are agents that have been demonstrated to affect EAAT2 expression and/or function, and their mechanism of action. EAAT2: Excitatory amino acid transporter 2; cAMP: Cyclic adenosine monophosphate; CREB: cAMP response element-binding protein; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; PI-3K: Phosphatidylinositol 3-kinase; PKA: Protein kinase A; EGF: Epidermal growth factor; PACAP: Pituitary adenylate cyclase-activating peptide; TGF-α: Transforming growth factor alpha; TNFα: Tumor necrosis factor alpha.

activate EAAT2 through the canonical NF-κB pathway[146]. However, NF-κB has also been shown to reduce EAAT2 transcription through co-activation of the TNFα-mediated NF-κB activation and N-myc activation, converting NF-κB to a repressor[145] (Table 1). One intriguing method of EAAT2 up regulation is through an as yet unknown mechanism driven by the presence of neurons or neuronal supernatant in cultured astrocytes, involving NF-κB and the kappa B-motif binding phosphoprotein (KBBP)[147]. Interestingly, in cultured astrocytes, transcription factor Ying Yang 1 appears to act as a repressor by recruiting histone deacetylases, coordinating an epigenetic method of silencing the gene[134]. Hypermethylation of EAAT2 promoter DNA is another epigenetic mechanism for EAAT2 down regulation that has been suggested[147,148].

Several post-translation modifications have been suggested to control EAAT2 levels and cellular localization. EAAT2 is internalized and degraded through Nedd-2 dependent ubiquitination at its C-terminus[149]. Sumoylation may also be involved, as non-sumoylated EAAT2 is primarily localized to the plasma membrane while only sumoylated EAAT2 is found in intracellular compartments[150] (Figure 1).

Functionally, EAAT2 has been show to exhibit reduced glutamate uptake when membrane cholesterol levels are reduced[130], as suggested by the inter-subunit crevices revealed by its structure. Additionally, certain exon-skipping EAAT2 splice variants appear to have an effect on EAAT2 function and surface localization[123].

In neuropathology, EAAT1 and EAAT2 have been shown to be reduced in several neurodegenerative disorders, such as amyotrophic lateral sclerosis, Alzheimer's disease, and Huntington's disease, where it was causally related to excitotoxic neuronal cell death[151]. In AUD, one genetic variant of human EAAT2, G603A, has been associated with antisocial[152] and cirrhotic alcoholics[153]. Rodent models, however, have provided an excellent tool for studying EAAT2 in AUD. The mouse EAAT2 gene (*slc1a2*) is located near a quantitative trait loci on chromosome 2 (E2)[125] that modulates seizure frequency and neuro-excitability in mouse models of epilepsy and alcohol withdrawal[154]. Many mouse studies have supported the involvement of EAAT2 in both the acute and chronic effects of ethanol, as well as the symptoms of withdrawal. Mulholland *et al*[155] showed disruption of NMDA and EAAT2 dependent Kv2.1 potassium channels by acute ethanol in the hippocampus. Several studies have demonstrated increased glutamatergic tone in regions of the cortico-striatal circuit[156], but there are only a handful of publications providing strong support for a correlating decrease in EAAT2. Increased EAAT2 expression and/or function alleviates withdrawal symptoms and reduces ethanol consumption[157,158], highlighting the importance of EAAT2 in regulating synaptic glutamate signaling.

CONCLUSION

According to the DSM-5® (diagnostic and statistical manual of mental disorders), alcohol use becomes a disorder when at least two of eleven specific criteria are met, including uncontrolled consumption, alcohol craving, or a development of tolerance. Addiction is a chronic, relapsing disorder characterized by the progression from occasional, controlled use to uncontrolled use and behavior over seeking and consumption, to chronic relapse after protracted abstinence. This process involves neuroplastic changes, primarily in the mesolimbic dopamine system, going from impulsive, positively reinforced behaviors (euphoria, social aspects) to compulsive, negatively reinforced behaviors (removing withdrawal symptoms)[10,159].

Understandably, early research in the field of addiction was focused on the ascending monoamine pathways of the dopaminergic reward system (mesocorticolimbic and extended amygdala), including the involvement of GABA and serotonin, all of which are

important in the early stages of addiction. In the past two decades, increased focus has shifted to glutamatergic signalling, as glutamate receptors in this system are greatly altered, and highly implicated in all stages of addiction, especially the later stages of dependence and withdrawal. The effects of alcohol on all types of glutamate receptors are under intense investigation, and much has been learned to date on how the dysregulated synaptic glutamate alters synaptic plasticity[66,67,86,121] (Figure 1). This shift in focus coincides with the success of clinical pharmacotherapies that alter the glutamatergic system. Acamprosate, a homo-taurine derivative similar in structure to GABA, especially when used with naltrexone, an opioid antagonist, has been one of the most effective drugs in preventing relapse in AUD[160]. However, the mechanistic understanding of these drugs is still not well understood. But, because of these relative successes, we are increasing our efforts in understanding synaptic glutamate modulation. For example, ceftriaxone, a beta-lactam antibiotic, has been shown not only to increase EAAT2 transcript levels, but also to attenuate ethanol consumption and withdrawal symptoms[157,158,161].

Regulation of EAAT2 and glutamate is complex, and understanding how to effectively control astrocytic EAAT2 function and/or expression could be the key to successfully treating AUD. By maintaining stable synaptic glutamatergic tone to ease withdrawal symptoms and allow re-establishment of non-pathological connections in the mesocorticolimbic circuit, recovery from addiction could be greatly ameliorated. In conclusion, a medication targeting an astrocytic glutamate transporter, especially EAAT2, will be an appropriate treatment option for AUD patients with pathologically increased glutamate levels.

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REVIEW

Cortical and subcortical gamma amino acid butyric acid deficits in anxiety and stress disorders: Clinical implications

Andrew W Goddard

Andrew W Goddard, Department of Psychiatry, UCSF Fresno Medical Education and Research Program, Fresno, CA 93701, United States

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Correspondence to: Andrew W Goddard, MD, Professor of Psychiatry, Department of Psychiatry, UCSF Fresno Medical Education Program, 155 N. Fresno Street, Fresno, CA 93701, United States. agoddard@fresno.ucsf.edu
Telephone: +1-559-4996580
Fax: +1-559-4996581

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Abstract

Anxiety and stress disorders are a major public health issue. However, their pathophysiology is still unclear. The gamma amino acid butyric acid (GABA) neurochemical system has been strongly implicated in their pathogenesis and treatment by numerous preclinical and clinical studies, the most recent of which have been highlighted and critical review in this paper. Changes in cortical GABA appear related to normal personality styles and responses to stress. While there is accumulating animal and human neuroimaging evidence of cortical and subcortical GABA deficits across a number of anxiety conditions, a clear pattern of findings in specific brain regions for a given disorder is yet to emerge. Neuropsychiatric conditions with anxiety as a clinical feature may have GABA deficits as an underlying feature. Different classes of anxiolytic therapies support GABA function, and this may be an area in which newer GABA neuroimaging techniques could soon offer more personalized therapy. Novel GABAergic pharmacotherapies in development offer potential improvements over current therapies in reducing sedative and physiologic dependency effects, while offering rapid anxiolysis.

Key words: Brain imaging; Anxiogenesis; Gamma amino acid butyric acid; Anxiety disorders; Anxiolysis

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Core tip: Preclinical and clinical studies strongly support the notion that impairments in gamma amino acid butyric acid (GABA) neurotransmission underpin human stress and anxiety disorders. Measurement of *in-vivo* brain GABA function with modern neuroimaging tools, such as proton magnetic resonance spectroscopy, in healthy and disease populations, has contributed greatly to this literature, and also offers the possibility of monitoring GABAergic anxiolytic therapy.

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INTRODUCTION

Anxiety and stress disorders are a major public health problem. They are the most common mental health conditions in the United States with a 12-mo prevalence rate of 18%[1]. Moreover, in their lifetime, over 25% of the United States population is expected to suffer from at least one anxiety disorder[1]. Anxiety disorders are responsible for long-term morbidity, and are now thought to be even more chronic than either substance use or mood disorders[2]. Similar observations have been reported from surveys conducted around the globe[3-5]. Across these studies, another consistent finding was the disproportionate impact of clinical anxiety on women. Finally, the societal and economic impact of anxiety syndromes is remarkable. In 1990, for instance, the direct and indirect cost to the United States economy due to these disorders was $42.3 billion[6].

Over the last three decades, diagnostic assessment and treatment options for morbid anxiety have improved considerably. Despite many theories, however, the pathogenesis of these conditions remains unclear. With a deeper understanding of fear and stress neurocircuitry, and the availability of more sophisticated imaging and genetic analytic tools, progress is being made. Within the field, there has been an emerging emphasis on the role of amino-acid neurochemical systems, such as the amino-butyric acid [The gamma amino acid butyric gamma acid (GABA), the major inbibitory neurotransmitter in the central nervous system (CNS)] and its excitatory counterpart, glutamate, in anxiogenesis and anxiolysis. This review will examine the evidence implicating abnormalities in GABA neurotransmission in the genesis of stress and anxiety in health and disease. Key anxiety and stress disorders such as panic disorder (PD), generalized anxiety disorder (GAD), and posttraumatic stress disorder (PTSD) will be reviewed through the lens of relevant animal models and human imaging studies implicating GABA deficits in anxiogenesis. The potential role of GABA in developmental anxiety will be mentioned, as will the evidence for GABA deficits in other neuropsychiatric syndromes in which anxiety is prominent. Finally, an overview will be provided of anxiolytic agents, which directly or indirectly support GABA neurotransmission, and which can address deficits in GABA functioning in the clinical disorders.

RESEARCH

A literature search was conducted using the PubMed and Thomson Web of Science v5.15 search engines. References were identified that directly related to the search terms, "GABA and clinical anxiety", including several review papers. Preference for inclusion in the current paper was given to articles published after 2009. However, some key/landmark papers published prior to 2009 were also included.

GABA NEUROTRANSMISSION AND NORMAL ANXIETY

Several rodent models have highlighted the role of the GABA synthetic isoenzymes glutamic acid decarboxylase 65 (GAD65) and GAD67 in the expression of normal mammalian fear. For example, knockdown of GAD67 protein in the mouse amygdala impaired normal fear extinction and decreased sensitivity to the benzodiazepine anxiolytic, diazepam[7]. In another experiment, a genetic impairment in GAD65 expression was linked to decreased GABAergic transmission and plasticity in the lateral amygdala (LA), which, in turn, was associated with generalization of conditioned fear responses[8]. A study of male rats additionally demonstrated the importance of the sex hormone, 17-estradiol, as a promoter of GAD65 expression, with pharmacologic inhibition of 17-resulting in increasing expression of anxious behaviors (decreased open field exploration)[9].

Improvements in proton magnetic resonance spectroscopy (^{1}H-MRS) technology and editing have led to the ability to quantify regional brain GABA concentrations, and other related amino acid metabolites, non-invasively. As a result, over the last 5 years, a number of studies applying these techniques in healthy humans have been published. These investigations have begun to define the relationships between normal human stress responses, personality type, and cortical GABA changes. For example, harm avoidance, as a normal human temperament trait, was observed to correlate positively with anterior cingulate cortex (ACC) GABA concentrations, and negatively with glutamate levels[10]. Evaluating extraversion/introversion and neuroticism in healthy subjects, another group reported a negative correlation between frontal GABA/creatine ratios (data acquired at 3 Tesla), and extraversion[11]. In another line of inquiry, acute psychological stress in healthy humans (threat of footshock) was associated with an acute decrease in prefrontal cortical GABA concentrations, similar to acute stress findings in the animal literature[12]. Investigating the impact of wellness interventions, such as yoga and walking, on cortical GABA, one group reported a relationship between improvements in stress level and mood, and increases in thalamic GABA levels, for yoga subjects only[13]. Other investigators using an fMRI/MRS assessment strategy, observed, in healthy humans, that lower insula cortex GABA levels and enhanced insula responses to interoceptive stimuli, together predicted higher levels of reported depressive affect[14]. However, studying female subjects, others did not observe a relationship between low insula GABA and inclination toward fearfulness[15].

Thus, cortical and subcortical GABA concentrations can be informative biological correlates of components of personality function and emotional processing, and appear to be change-sensitive markers of normal responses to acute stress and relaxation. It is foresee-

able that routine assessment of inhibitory brain function in this manner is likely to enhance the effectiveness of early-intervention and prevention protocols designed to interrupt the genesis of chronic anxiety or depressive states.

GABA DEFICITS IN PD

Although the neurobiological mechanisms underlying this common and disabling psychiatric syndrome remain unclear, a range of preclinical and clinical findings have implicated disturbances in GABA function in its pathophysiology. Animal modeling work[16] has demonstrated that biochemically-induced GABA deficits in the dorsomedial hypothalamus (DMH) of rats predispose to sodium lactate-induced panic, also an important clinical feature of human PD[17]. Follow-up work with this particular model has observed that lactate sensitivity and other anxious rodent behaviors could be driven by loss of GABAergic inhibition to a local DMH and perifornical population of peptidergic orexin (ORX) neurons[18]. Thus, impaired GABA function may facilitate ORX neuronal hyperactivity, thereby leading to increased sympathetic activation, and panicogenesis.

Other animal models of chronic anxiety/panic have focused on deficits in functioning of synaptic $GABA_A$ receptors as a risk factor for anxiety-proneness. For example, genetically induced deficits (moderate reductions) in expression of $GABA_A$ receptor 2 subunits (by heterozygous knockdown or knockout), were associated with neophobic behaviors, behavioral inhibition, or exaggerated defensive responses to mild threat[19]. More recently, the same group demonstrated that 2-containing $GABA_A$ receptor subpopulations are also implicated in the defensive response to mild threat, in that mice lacking 2 subunits exhibited anxious phenotype[20]. The animal models above also have parallel human findings, which we will now mention.

Deficits of GABA neuronal functioning have been implicated in the pathophysiology of PD by recent ^{1}H-MRS[21-23], $GABA_A$-benzodiazepine receptor single photon emission computed tomography (SPECT)[24] and positron emission tomography (PET) studies[25,26]. Not unlike the lactate-sensitive animals referred to earlier, humans with PD have been reported to have cortical GABA deficits in occipital, ACC/medial prefrontal cortex (mPFC), and basal ganglia regions of interest, though one MRS-GABA study of the prefrontal cortex was negative[27] (Table 1 for additional details). Similar GABA deficits (also identified by MRS) have been reported in other human anxiety spectrum disorders, such as social anxiety disorder[28] and obsessive compulsive disorder (OCD) (thalamic and mPFC deficits respectively)[29]. If GABA deficits in humans with PD also extend to impairment of GABAergic inhibition of DMH ORX neurons, this could account for spontaneous or lactate-induced panic in PD patients and in other anxiety patients who experience panic. Other domains of PD symptomatology, such as neophobia, anticipatory fear, and phobic avoidance, could conceivably be more related to the cortical deficits in $GABA_A$ receptor status identified by the PET and SPECT investigations above.

Furthermore, low cortical GABA in PD might be a trait-like entity, since neither acute nor chronic administration of anxiolytic pharmacotherapy was associated with reversal of these deficits[30]. Thus, low cortical GABA could be an important ongoing vulnerability factor conferring panic-proneness[31]. Moreover, in a retrospective analysis of one data set, the presence of a mood or anxiety family history appeared related to the magnitude of cortical GABA deficits observed in PD[32]. Low cortical GABA therefore has potential as a biomarker for PD and related stress conditions.

Human genetics studies of GABA and PD

The familiality and heritability of PD have been well established. Based on a heritability estimate of 43%[33], genetic factors are a significant contributor to the pathogenesis of PD. However, despite extensive clinical investigations in a number of anxiety disorders, the question remains open as to which genes are critically involved in anxiogenesis[34]; this is likely due to the fact that the "genetic architecture" of PD, similar to other high-prevalence medical conditions, is complex and attributable to multiple genes of small effect. The GABA neuronal system, though, continues to be a logical candidate system for future genetic studies of PD because of the current clinical neurobiological data (reviewed above) implicating GABA abnormalities in this condition, as well as the effectiveness of established and promising GABAergic therapies (Table 2).

Despite the recent positive $GABA_A$ receptor PET imaging findings in PD, work evaluating the potential role of $GABA_A$ receptor genes (*GABRA2*, *3*, *6*, and *GABRAG2*) in anxiety spectrum disorders has thus far been negative[35]. Other studies have focused on the genes *GAD1* and *GAD2*, which code for the GABA synthetic isoenzymes, GAD_{67} (found throughout the neuron), and GAD_{65} (found more in axon terminals, and related to regulation of short-term demands for GABA), respectively. In the investigation of Hettema *et al*[36] 2006, involving 589 patients and 539 controls, the data suggested an association of several SNPs of the *GAD1* gene with the personality trait of neuroticism (N), a risk factor for both anxiety disorders and major depression (MDD). A more recent case-control study, in a cohort of n = 268 anxiety patients, n = 542 MDD patients, and n = 541 healthy subjects, identified an association between elevated levels of behavioral inhibition trait (BI) in the patient groups, and several *GAD2* gene SNPs[37]. Finally, an association study in a cohort of n = 238 anxiety patients (84% with PD), and n = 267 healthy subjects recently linked several SNPs (rs2930152, rs2697153, and rs956053) of the GAT1 transporter gene, SLC6A1, with panic attacks. The odds ratio of the association increased in more severely

Table 1 Neuroimaging studies of gamma amino acid butyric acid function in anxiety disorders

Method	Finding	Design	Clinical significance	Ref.
^{123}I-Iomazenil SPECT	Decreased L hippocampus and precuneus $GABA_A$ R binding	Parallel gp, 13 PD patients *vs* 16 healthy control subjects (HCs)	Chronic stress due to active PD can result in impaired limbic processing *via* reduced $GABA_A$ receptor density or function	Bremner *et al*[24]
^{1}H-MRS	Decreased OC GABA in PD	14 PD *vs* 14 matched HCs (historical)	Could be related to impaired production of GABA or of GABA-glutamine cycling in PD	Goddard *et al*[21]
GABA, 1.5T	No change in OC GABA pre and post-acute and chronic BZD Rx	10 PD *vs* 9 HCs	Suggests low GABA is a trait-like biomarker for PD	Goddard *et al*[30]
^{1}H-MRS GABA, 3T	Decreased ACC and BG GABA in patients	22 PD (medicated) *vs* 24 matched HCs, single voxel study	Impaired top-down inhibition of limbic activity in PD	Ham *et al*[23]
^{11}C-flumazenil PET	Reduced $GABA_A$ R binding in L and R insula Cx of PD	11 PD *vs* 21 HCs	Interoceptive/somaticsensitivity in PD likely could be insula-mediated	Cameron *et al*[25]
^{1}H-MRS-GABA, 4T	Reduced thalamic GABA in SAD	10 SAD patients *vs* 10 matched HCs	Impaired GABA function in thalamus in SAD could affect social cognition *via* amplification of external threat cues	Pollack *et al*[28]
^{11}C-flumazenil PET	Reduced frontal, temporal, parietal Cx $GABA_A$ binding pot. in PD	15 BZD-naïve PD *vs* 18 HCs	Generalized cortical impairment in $GABA_A$ function in PD could be a cause or effect of PD. If endogenous BZD-like ligands overproduced could be evidence of a compensatory response to chronic stress	Hasler *et al*[26]
^{1}H-MRS-GABA, 3T	Normal PFC GABA in PD	Parallel gp 17 PD *vs* 17 sex-matched HCs	In contrast to previous + results in ACC and OC ROIs	Hasler *et al*[27]
^{1}H-MRS-GABA, 3T	Reduced mPFC GABA in OCD	24 OCD patients *vs* 22 matched HCs	Could contribute to cortical-striatal circuit dysfunction in OCD	Simpson *et al*[29]
^{1}H-MRS-GABA, 3T	Reduced ACC/mPFCx GABA in PD	Parallel gp 11 PD *vs* 8 matched HCs	Effect size greater in FHx+ PD ACC GABA negatively correlated with enhanced ACC-precuneus, connectivity, 2 DMN nodes	Long *et al*[22] Shin *et al*[70]
^{1}H-MRS-GABA, 4T	Low R A^r insula Cx GABA in PTSD	13 PTSD patients *vs* 13 matched HCs	Relationship btw low insula GABA and higher state-trait anxiety levels	Rosso *et al*[50]
^{1}H-MRS-GABA, 4T	Lower GABA in tempo-parietal Cx and occipital-parietal Cx in PTSD	27 PTSD patients *vs* 18 trauma-exposed controls	Low GABA finding mediated by high levels of insomnia	Meyerhoff *et al*[51]
^{1}H-MRS-GABA, 3T	Elevated DLPFC and ACC GABA and glutathione levels	12 PTSD patients *vs* 17 non-PTSD trauma controls	Oxidative stress implicated in the pathophysiology of PTSD, as well as elevated prefrontal inhibitory neurotransmission	Michel *et al*[52]

SAD: Social anxiety disorder; PTSD: Post-traumatic stress disorder; GABA: Gamma amino acid butyric acid; mPFC: Medial prefrontal cortex; PET: Positron emission tomography; FDG: Fluorodeoxyglucose; PGB: Pregabalin; ACC: Anterior cingulate cortex; BZD: Benzodiazepine full agonist; MRS: Magnetic resonance spectroscopy; CNS: China national standards.

ill patients (frequent panickers), reaching a value of 2.5[38]. However, none of these studies assessed cortical GABA as part their study design. One recent investigation, employing this strategy to explore 5-HT/GABA interactions as a risk factor for panic/anxiety disorder, reported an association between (higher) prefrontal GABA concentrations and presence of a tryptophan hydroxylase isoform 2 gene polymorphism, especially in female mood/anxiety patients. This polymorphism had been previously linked to decreased TPH2 mRNA expression in PD. Follow-up studies are needed to confirm this association due to the small subgroup size of patients studied; however, this line of inquiry is exciting given the high rate of women affected by PD. There is also preliminary evidence of *GAD1* gene hypomethylation as a potential epigenetic response to negative life stressors in PD[39]. This is of significance clinically due to the close association of life events and onset of PD illness episodes, and in view of the MRS data suggesting that low cortical GABA is a risk factor for panic-proneness.

GAD/TRAIT ANXIETY AND GABA

Several animal models have linked perturbations in GABA function to elevated trait anxiety. For example, mice bred for high anxiety behaviors (HAB), compared to control animals, were found to have a complex pattern of intra-amygdala GABA neuronal changes[40]. The amygdala has been identified as a key fear-processing structure within the fear circuit. Levels of GAD65 and GAD67 mRNA and protein were elevated in the basolateral amygdala (BLA) in HAB animals *vs* controls. In addition, mRNA expression of $GABA_A$ receptor subunits 1, 2, and 2 in the BLA was increased in HAB mice, while transcription of 5 and 1 subunits was reduced in the central and medial amygdala. Also, BLA levels of FosB, a marker for neuronal activation,

Table 2 Gamma amino acid system butyric acid effects of psychotropics with anxiolytic activity

Drug	Effect on GABA system	Test paradigm	Comments	Ref.
Benzodiazepines	Agonists at $GABA_A$ receptor complex	Alprazolam blocked ACC activation induced by CCK4 in controls.	fMRI study in 16 healthy male subjects	Leicht *et al*[71]
		Preclinical study of BZD effects on conditioned and unconditioned fear in mice	α2 subunit-containing $GABA_A$ receptors sufficient for BZD anxiolysis of unconditioned fear. α1 and α2 subunits needed to suppress conditioned fear	Smith *et al*[72]
SSRIs	Increased cortical GABA levels	Clinical MRS-GABA in MDD patients		Sanacora *et al*[73]
	Increased neuroactive steroid levels in the CNS	CSF ALLO increases in SSRI treated MDD patients (n = 15)		Uzunova *et al*[74]
TCAs	Increased release of GABA	Preclinical; desipramine effects studied		Korf *et al*[75]
MAOIs	Increase total brain GABA	Preclinical study of phenelzine effects		Paslawski *et al*[76]
Gabapentin	Increased cortical GABA	MRS-GABA in seizure patients	Chronic medication admin	Petroff *et al*[77]
PGB	Increased release of GABA	PGB decreases activation of left insula and amygdala in response to emotional images	fMRI activation study in 16 healthy humans	Aupperle *et al*[78]
Tiagabine	Blocks GAT-1 and inhibits synaptic GABA reuptake	FDG-PET study pre and post a 6-week tiagabine trial for social phobia	15 social anxiety disorder patients and 10 controls. vmPFC metabolism increased with treatment	Evans K *et al*[79]
Vigabatrin	Blocks GABA transaminase	Blocks CCK4 panic in healthy humans	Not clinically available due to ocular AEs	Zwanger *et al*[80]

GABA: Gamma amino acid butyric acid; mPFC: Medial prefrontal cortex; PET: Positron emission tomography; FDG: Fluorodeoxyglucose; PGB: Pregabalin; ACC: Anterior cingulate cortex; BZD: Benzodiazepine full agonist; MRS: Magnetic resonance spectroscopy; CNS: China national standards.

were notably increased. This pattern of findings in HAB animals can be interpreted as evidence of excessive excitation in the BLA due to loss of inhibitory GABA tone from the central and medial nuclei, with compensatory upregulation of BLA GABA synthetic enzymes. In another study, liver X receptor knockout mice were noted to exhibit anxious behaviors and to have reduced expression of GAD65 and 67 enzymes in the ventro-mPFC[41]. Anxiety in this protocol could have been mediated by loss of ventromedial prefrontal inhibitory GABA tone to the amygdala. Chronic anxiety in rodents was induced by inhibition of GABA synthesis in the bed nucleus of the stria terminalis area of the extended amygdala, a model reminiscent of human GAD, since these animals not only had persistent anxiety, but were also lactate-insensitive[42].

Thus far, there have been no clinical studies of cortical GABA levels or $GABA_A$ receptor binding in GAD, and, to date, genetic association studies of $GABA_A$ receptor subtypes have been negative[35]. Of interest clinically are studies which provide indirect evidence of excessive amygalar excitability in GAD patients. In one fMRI study comparing GAD patients and healthy subjects, neutral and threat cues triggered excessive amygdalar activation responses in GAD subjects[43]. This result is consistent with of an excitation/inhibition imbalance (glutamate/GABA function imbalance) within the amygdala in GAD, and is also consistent with the animal model findings presented above.

PTSD AND GABA FUNCTION

Animal studies

An unpredictable stress paradigm (unpaired odor-shock administration) in neonatal rodents, was associated with anxiety phenotypic behavior in adulthood, together with amygdalar upregulation of genes related to synaptic transmission, such as serotonin (5-HT) and *GABA* genes[44]. In another rodent study focusing on inescapable stress (inescapable footshock), and examining morphological and neurochemical changes in the prefrontal cortex and hippocampus, post-stress hippocampus cell damage was observed and found to be related to a glutamate/GABA neurochemical imbalance[45]. One group has recently developed a PTSD mouse model by inducing a null mutation in the *GAD65* gene. The GAD65 enzyme, as noted previously, is critically involved in activity-dependent regulation of GABA release, and is necessary for proper maturation of the GABA system in adolescence. Mutant mice had hyperexcitability of the amygdala and hippocampus, increased anxiety, and pathological fear memory, all features of human PTSD[46]. In contrast, a GAD65 haplo-deficiency animal model was associated with delayed elevations in cortical and limbic GABA levels, yet was found in these animals to confer resilience to the effects of stress[47]. In another animal model of chronic stress (immobilization/restraint stress), tonic (but not phasic) $GABA_A$ receptor currents in the amygdala were observed to be persistently reduced following this type of stress, resulting in excessive amygdalar excitability[48]. Such enduring, stress-related changes could be relevant to the development of human anxiety and stress disorders. Furthermore, chronic restraint stress in high-anxiety animals in one study led to decreased brain (cortex and hippocampus) expression of 2 subunits of the $GABA_A$ receptor, suggesting a role for these changes in HPA axis dysfunction (upregulation) and stress

symptomatology[49].

Several MRS-GABA studies have also documented abnormalities in cortical GABA in human PTSD (Table 1). For example, insula cortex GABA levels in PTSD patients were recently found to be decreased[50]. In another protocol, combat PTSD patients *vs* non-combat controls exhibited abnormally low levels of parieto-occipital and temporal cortical GABA, which were accounted for by insomnia severity[51]. Yet another group has reported abnormal increases in prefrontal cortical GABA and glutathione in PTSD, implicating oxidative stress and possibly increased frontal cortical inhibition in PTSD pathophysiology[52].

Clinical significance

From the available data it is unclear whether deficits in GABA neurotransmission are a risk factor for PTSD, or are mainly the result of chronic stress, and the associated symptoms of PTSD. It is also unclear whether GABA deficits/dysfunction in PTSD could be the result of compensatory responses to stress that have become depleted, or whether they are particularly involved in the perpetuation of components of chronic PTSD (*e.g.,* hyperarousal, cue-sensitivity, increased startle).

SEPARATION ANXIETY AND GABA

Animal work has implicated the GABA system in unexpected ways in the behavioral and neurochemical response to maternal separation (MS). In one investigation, MS, in addition to promoting anxiety behaviors, enhanced tonic GABA currents in cortical layer 5 pyramidal neurons (juvenile rats), and promoted subsequent neurogenesis (subventricular zone, cortical layer 1, and dentate gyrus), and differentiation into GABA neurons (adult rats)[53]. These persistent brain changes might, in turn, predispose to later-life behavioral disturbances. Some investigators have noted that MS during breast-feeding produced behavioral changes and $GABA_A$ receptor 1 subunit expression changes that were gender-dependent[54]. In this study, MS male rats *vs* controls tended to exhibit less exploratory behavior, and have less 1 subunit expression in the amygdala, mPFC, and paraventricular nucleus (PVN). Females, however, had more exploratory and head-dipping behaviors *vs* controls, and less subunit staining in the mPFC, PVN, preoptic area, and hippocampus. These results highlight the possibility of gender difference in mechanisms of anxiogenesis. This line of inquiry may well improve our understanding of gender differences in the expression of human anxiety syndromes. A recent review of biological underpinnings of critical periods in fear learning and memory encoding highlighted the potential role of the GABA system on neuroplasticity in the peri-adolescent period. Adolescence is a time when GABA neurotransmission is rapidly improving in efficiency, and disruption in these often nonlinear developmental processes could predispose to anxiety and mood pathology in the adolescence and beyond[55]. To date, there has been little work in humans directly exploring the impact of developmental events/stressors on GABA function, but this could be a fruitful area of investigation.

GABA DEFICITS AND ANXIETY IN OTHER NEUROPSYCHIATRIC DISORDERS

Studies of other pathological conditions with anxiety as a feature [traumatic brain injury (TBI), temporal lobe epilepsy (TLE), and depression (MDD), have also suggested a relationship between impaired GABA function and anxious behavior. Using a rodent model of mild TBI (mild controlled cortical impact), experimenters reported trauma-related increases in anxiety, and reduced BLA GABA function (decreased GABA cell numbers) leading to BLA hyper-excitability[56]. In TLE patients with mood or anxiety syndromes, together with several other GABA system changes, temporal lobe tissue levels of GABA were noted to be lower *vs* autopsy controls[57]. Finally, in a CSF assessment study of unmedicated MDD patients, those with anxious features tended to have abnormally low CSF GABA levels in contrast to patients without anxious features[58]. Thus, abnormal GABA function could be an important mediator of anxiety across multiple neuropsychiatric syndromes, and these preliminary data suggests the potential efficacy of GABAergic pharmacotherapies to stabilize this symptom cluster (Table 2).

DISCUSSION/TREATMENT IMPLICATIONS

Table 1 summarizes the human neuroimaging studies implicating GABA neuronal dysfunction in anxiety and stress disorders. The majority of the studies, utilizing ^{1}H-MRS techniques, have reported cortical or subcortical GABA deficits in structures relevant to the fear circuit across several different diagnoses. Most studies, however, have been conducted with relatively small samples. The PTSD findings should be interpreted with caution as other factors such as state anxiety, and insomnia appear to be mediating some of the GABA changes reported. In the case of PD, there were 3 positive findings (2 in the ACC/mPFC and 1 in the OC), and one negative finding (prefrontal cortex). One of the ACC studies (Ham *et al*[23]), however, was conducted in medicated PD patients, and hence it is impossible, in this instance, to attribute the GABA changes to the PD diagnosis. The ability to look at interrelated amino-acid metabolites (GABA, glutamate, glutamine) can add power to a study, as demonstrated in the OCD report of Simpson *et al*[29] where impaired mPFC GABA inhibition of subcortical structures was associated with elevated glutamate/glutamine levels in the thalamus. The majority of studies provided a baseline GABA assessment, and therefore, while suggestive of an

association with a specific disorder, at this stage of the research, the idea that the findings indicated effects of chronic stress cannot be ruled out totally. One exception was one of the PD studies in which acute and chronic benzodiazepine medication effects were prospectively measured, and which suggested loss of normal acute GABA counter-regulatory mechanisms, and tonically low GABA in PD[30]. A limitation of this study was the selection of the OC ROI, which is not directly related to fear-processing circuitry. While there is more consistency with the $GABA_A$ receptor findings in PD, again cause-effect relationship cannot readily be disentangled with the study designs used. Future study designs are likely to benefit power-wise from a careful assessment of family history status[32], and the use of a combination of functional imaging techniques (*e.g.,* fMRI or fcMRI together with MRS-GABA assessments), as well as the use of more dynamic MRS approaches (*e.g.,* 13C-labelled glucose/MRS evaluations assessing neuronal and glial contributions to the total GABA pool). The use of more dimensional approaches to anxiety psychopathology classification, as proposed in the NIMH RDoC project, may improve consistency of results (*e.g.,* studying acute responses to fear *vs* anticipatory fear across a range of DSM-V anxiety conditions). Finally, there are important limitations with the GABA neuroimaging paradigms reviewed. MRS evaluations of GABA offer an integrated assessment of intra-neuronal GABA in a large ROI, while current SPECT and PET methodologies offer the ability to study post-synaptic $GABA_A$ receptor status. However, the ability to adapt PET methodology to study the intra-synaptic fraction of GABA, as recently reported[59], now permits a more comprehensive evaluation of GABA neurotransmission across neuropsychiatric disorders.

GABA AND ANXIOLYTIC TREATMENT MECHANISMS

The GABA system has been implicated in the therapeutic mechanism of action of a number of psychotropic agents with anxiolytic activity (Table 2). Benzodiazepine full agonists (BZDs) are the prototypical class of agents in this respect, and their allosteric enhancement effect at the BZD site of $GABA_A$ receptor complex is well known[60]. Preclinical work has further defined the role of discrete $GABA_A$ receptor subunits in the separate clinical effects of the BZDs such as anxiolysis, sedation, muscle relaxation, and anticonvulsant effects. The 2 subunit for instance, is necessary to the anxiolytic action of BZDs[60]. In contrast, sedative, anticonvulsant, amnestic, and dependency effects in general require the presence of the 1 subunit[61]. Antidepressant agents also have the capacity to facilitate GABA function *via* augmentation of GABA levels, and neurosteroid levels. Also, newer-generation GABAergic anticonvulsant medications have begun to demonstrate anxiolytic effects, in parallel with localized physiological changes within the fear circuit. From the overview provided in Table 2, enhancement of GABA neurotransmission might be viewed as a final common pathway of anxiolytics in general, or at least a key pathway for anxiolysis. If, as the literature currently suggests, GABA neuronal deficits/abnormalities are present in a range of clinical anxiety conditions, then GABA enhancers of different types might be expected to offset these deficits, thereby promoting anxiolysis, and restoration of function. The future prospect of more predictive and personalized anxiety treatment planning and monitoring is also attainable given the availability of imaging tools that can reliably measure cortical GABA, and other amino-acid metabolites. In the specific case of PD, where cortical GABA level deficits may be trait-like, it would be of interest to know if long-term GABA deficits persist, or whether they resolve at some point during maintenance treatment. "Normalization" of CNS GABA levels might be a more appropriate end point/cue to taper psychotropic treatment when combined with more traditional clinical indices of remission.

NOVEL ANXIOLYTICS TARGETING GABA

The GABA system provides a rich array of molecular targets for ongoing drug development initiatives, offering hope for stress/anxiety conditions in which GABA impairments are implicated. Considerable effort has been devoted to the enterprise of generating non-sedative anxiolytics, based on targeting selective $GABA_A$ receptor subunits. While, in this regard, 2/3 subunit-selective compounds have shown much promise in the lab[62], translation to the clinical has been limited by adverse events (*e.g.,* liver toxicity)[63]. More recently, attention has focused on subunit-selective compound development with both preclinical[64] and clinical progress being made[65]. The latter agent, etifoxine, exhibits a dual mechanism, 2/3 subunit selective agonism and neurosteroidogenic stimulation, to enhance GABA neurotransmission. A novel molecular target for ligand development, a mitochondrial Translocator Protein (18 kD), regulates the initial and rate-limiting step in neurosteroidogenesis[66]. Neuroactive steroids synthesized from this pathway, such as allopregnenolone, act as positive allosteric modulators at a specific neurosteroid site within the $GABA_A$ receptor complex. Positive synthetic ligands at this site include the agent XBD173, which showed preliminary clinical benefit for panic anxiety, and YL-IPA08, which displayed anxiolysis in a PTSD animal model[67]. In addition, a synthetic neurosteroid analog, ganaxolone, has shown therapeutic potential in a mouse model of PTSD[68]. Within the glutamate system, bilateral intra-amygdala (BLA) administration of the GluK1 kainate receptor agonist ATPA, facilitated GABA neurotransmission to promote anxiolysis in one animal model of stress[69].

CONCLUSION

Anxiety and stress disorders are a major public hea-

lth concern. However, their pathophysiology is still unclear, and requires ongoing investigation. The GABA neurochemical system has been strongly implicated in their pathogenesis and treatment by numerous preclinical and clinical studies, the most recent of which have been highlighted in this review. Changes in cortical GABA appear related to normal personality styles and normal responses to stress. While there is accumulating *in-vivo*, neuroimaging evidence of cortical and subcortical GABA deficits across a number of anxiety conditions, a consistent pattern of findings in specific brain regions for a given disorder is yet to emerge. Neuropsychiatric conditions with anxiety as a clinical feature may have GABA deficits as an underlying feature. Different classes of anxiolytic therapies appear to support GABA function, and this may be an area in which newer GABA neuroimaging techniques could soon offer more personalized therapy. Novel GABAergic pharmacotherapies in development offer potential improvements over current therapies in reducing sedative and physiologic dependency effects, while offering rapid anxiolysis.

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REVIEW

Sex differences in cognitive impairment in Alzheimer's disease

Keith R Laws, Karen Irvine, Tim M Gale

Keith R Laws, Karen Irvine, Tim M Gale, School of Life and Medical Sciences, University of Hertfordshire, Hatfield, Hertfordshire AL10 9AB, United Kingdom

Karen Irvine, Tim M Gale, Hertfordshire Partnership University NHS Foundation Trust, the Colonnades, Beaconsfield Close, Hatfield, AL10 8YE, United Kingdom

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Correspondence to: Keith R Laws, PhD (CANTAB), Professor, School of Life and Medical Sciences, University of Hertfordshire, College Lane, Hatfield, Hertfordshire AL10 9AB, United Kingdom. k.laws@herts.ac.uk
Telephone: +44-1707-281137

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Abstract

Sex differences in neurocognitive abilities have been extensively explored both in the healthy population and in many disorders. Until recently, however, little work has examined such differences in people with Alzheimer's disease (AD). This is despite clear evidence that AD is more prevalent in women, and converging lines of evidence from brain imaging, post-mortem analyses, hormone therapy and genetics suggesting that AD affects men and women differently. We provide an overview of evidence attesting to the poorer cognitive profiles in women than in men at the same stage of AD. Indeed, men significantly outperform women in several cognitive domains, including: Language and semantic abilities, visuospatial abilities and episodic memory. These differences do not appear to be attributable to any differences in age, education, or dementia severity. Reasons posited for this female disadvantage include a reduction of estrogen in postmenopausal women, greater cognitive reserve in men, and the influence of the apolipoprotein E ε4 allele. Assessment of cognitive abilities contributes to the diagnosis of the condition and thus, it is crucial to identify the role of sex differences if potentially more accurate diagnoses and treatments are to emerge.

Key words: Dementia; Gender; Sex differences; Cognition

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Core tip: This review assesses evidence that women with Alzheimer's disease (AD) show greater cognitive impairment than men. The evidence shows that female AD patients are outperformed by males in multiple cognitive domains including visuospatial, verbal processing, semantic and episodic memory. This disadvantage is not attributable to sex differences in age, education level, or dementia severity. Possible explanations include estrogen loss in women or a greater cognitive reserve in men, which may provide protection against the disease process. Such findings have implications for tailoring more specific gender-based treatments.

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INTRODUCTION

"Biomedical research in general, and neuroscience in particular, has been built on a false assumption that one may safely ignore potential sex influences"[1].

Alzheimer's disease (AD) is the most common neurodegenerative disease associated with aging, with worldwide estimates of 30 million people with dementia, 4.6 million new cases annually, and one new case every 7 s[2]. The prevalence and the incidence of AD are greater amongst women than men and this discrepancy increases with advanced age[3-5].

A meta-analysis of 13 population studies from across United States, Europe, and Asia indicates that women are at significantly greater risk of developing AD, though not other dementias[6]. The neurocognitive profiles of male and female AD patients, however, are less well-described. These profiles require far greater examination as sex differences in the pattern of cognitive decline may yield vital information about differential risk factors, pathogenesis and most importantly, treatment of AD in men and women. In order to make sense of any sex differences that emerge under AD neuropathology, we first need to have a clear understanding of those sex differences that may already exist in the normal population, and specifically the healthy elderly population.

SEX DIFFERENCES IN THE GENERAL POPULATION

Sex differences in cognitive abilities receive consistent, extensive discussion in the "normal" literature, with the prevailing view being that women tend to have better verbal abilities[7,8], while men display a visuospatial advantage[8,9].

VERBAL ABILITIES

Tasks measuring verbal ability generally rely on rapid access to semantic and phonological information. Category fluency requires a person to name as many exemplars of a category (*e.g.*, tools) as possible, usually within one minute, while lexical fluency involves listing as many words beginning with a given letter (typically, F, A and S). In confrontation naming, the respondent must produce names for a series of visually presented items, usually but not exclusively line drawings. This task is regarded as a measure of core semantic memory function, though performance depends also on visual acuity and retrieval of phonological information.

In their meta-analysis of sex differences in almost 1.5 million participants, Hyde *et al*[7] found a small, but reliable female advantage in verbal ability with a *Cohen's d* = 0.11 (Cohen's *d* is calculated from: Mean of group A - Mean of group B/pooled standard deviation of A and B); and effect sizes are traditionally viewed as small (*d* = 0.2), medium (*d* = 0.5) or large (*d* = 0.7+). The small effect size reflects the mixed findings, with 44 studies (27%) reporting females outperformed males, 109 (66%) found no significant difference, and in 12 (7%) males outperformed females. Recent evidence suggests that sex differences in verbal ability are not universal but task-dependent. For lexical fluency, a female advantage has emerged in some studies[10,11], but not others[12,13]. Category fluency tasks do not elicit reliable sex differences[14] although differences may emerge for specific categories; for example, a female advantage for fruits and a male advantage for tools[15]. Confrontation naming, by contrast, has shown a male advantage in the few published papers examining sex differences[16]. In summary, sex differences in verbal abilities in the general population may be smaller than once believed[17].

VISUOSPATIAL ABILITIES

Linn *et al*[18] partitioned visuospatial tasks into three groups: Mental rotation, spatial perception (spatial working memory) and spatial visualisation (navigation). Mental rotation involves the ability to mentally rotate a two or three-dimensional figure rapidly and accurately. Spatial perception tasks are those where participants are required to determine spatial relationships with respect to the orientation of their own bodies in spite of distracting information. Spatial visualisation involves complicated multi-stage manipulations of spatially presented information (*e.g.*, the embedded figures test).

Sex differences in normative visuospatial processing are more robust than those documented for verbal processing. Voyer *et al*[9] meta-analysed 286 studies spanning 50 years, finding that men significantly outperformed women on most visuospatial tasks. Within Cohen's[19] nomenclature, the effect sizes were large for mental rotation (*d* = 0.73), medium for spatial perception (*d* = 0.44), and small for spatial visualization (*d* = 0.13). A later review by Li *et al*[20] examined 16 studies comparing men and women on spatial rotation and 11 on spatial navigation, and all 27 pointed to a male advantage.

AGE RELATED COGNITIVE DECLINE

As expected, many aspects of cognitive performance do decline with age[21-24]. Although not typically apparent for category fluency[25] and spatial perception[26], age-related deficits are seen in lexical fluency[27] and block design tasks[28] - underscoring how the precise requirements of the verbal and spatial tasks are crucial.

Greater age-related cognitive decline in healthy

men than women has been established not only in cross-sectional studies, but also crucially in longitudinal studies[29-34]. For example, Wiederholt *et al*[33] assessed 1692 participants aged 55-94 years finding that performance on all cognitive tests decreased progressively with age, but that the decline was slower in women. More recently, using internet testing, Maylor *et al*[23] examined sex differences and age-by-sex differences on various cognitive tasks in a very large sample of healthy individuals (109612 men and 88509 women). Importantly, and consistent with other studies, men showed greater age-related decline than women, irrespective of whether the task was one on which they were better. Of course, such large samples are sufficiently powered to detect even the most trivial differences and although the age by gender interactions described in this study are highly significant, they account for just 0.1% of the variance in cognitive performance.

As part of the Whitehall Ⅱ cohort study, Singh-Manoux *et al*[35] assessed 5198 male, and 2192 female, civil servants, aged 45-70, monitoring their cognitive performance (memory, vocabulary, reasoning and verbal fluency) over 10-years. Their results suggest that cognitive performance may decline earlier than previously thought. For example, reasoning scores decreased by 3.6% for men and women aged 45-49, and 9.6% and 7.4% respectively for those aged 65-70. The authors also noted the much larger cross-sectional than longitudinal age-related decline in women, which they attributed to cohort differences in education, with older women tending to be less well educated.

Based on neurocognitive and behavioural data, some have proposed that "age is kinder to women"[36]; however, any such female advantages are quite small and contentious. Meinz and Salthouse[36] meta-analysed 25 studies (5201 individuals aged 18-64) investigating sex differences in the patterns of age-cognition relations across a wide range of tasks. They concluded that "large (12%-25% of the total variance) effects related to age, small (at most 3% of the variance) effects associated with gender, and small to non-existent (less than 1%) effects associated with interactions of age and gender on measures of cognitive performance" (p 63). Only a minority of measures (speed and reasoning) revealed significant age by sex interactions: Men had smaller age-related declines. Although the age-related differences across sex were quite small, favouring women (only spatial abilities demonstrated a large sex gap favouring men), the confidence intervals for estimated effect sizes were small, suggesting the results are reliable.

Other research, however, suggests that men and women decline at similar rates[22,27,37,38]. Gerstorf *et al*[22] examined 368 participants aged 70-100 over a 13-year period, finding that for all cognitive tests men and women declined in parallel. Proust-Lima *et al*[38] however, found that after adjusting for vascular status, sex differences in cognitive decline did emerge, but only at the oldest age, with women showing a steeper decline than men do. Similarly, in a sample of 647 twin pairs (both dizygotic and monozygotic) aged between 65 and 98 years, Read *et al*[24] described larger sex differences in working memory and perceptual speed deficits at later ages, with women faring worse.

Therefore, although age-related cognitive decline is evident on many tasks, the weight of evidence points to similar levels of impact in men and women until the very oldest age, when women suffer a faster decline. We might then expect to find that sex differences in the younger population persist in the elderly. Furthermore, any sex effects in AD patients that differ from that seen in the healthy population are likely to be due to the disease process rather than aging *per se*.

SEX DIFFERENCES IN THE ELDERLY

Research suggests that typical sex differences in cognitive performance may persist into old age, with better visuospatial and language skills in males and females respectively[11,23].

VERBAL ABILITIES

The pattern of sex differences in category fluency of the healthy elderly is far from consistent, with some finding a female advantage[11,39-44], others no sex differences at all[22,25,45-48] and at least one report of a male advantage[33]. The presence of an effect may depend upon the specific category examined, with reports of both a female advantage and an absence of sex differences[49] or both a female advantage and a male advantage[41] or all three possibilities depending upon the specific semantic category examined[15].

A picture naming advantage in elderly males has been documented[16,50-53] mirroring the advantage often seen in young adults, although not in all studies of the elderly[16,39,43-46,54-59]. In a longitudinal study by Connor *et al*[16], the rate of decline in naming was comparable for men and women.

VISUOSPATIAL ABILITIES

Corresponding to the findings in the healthy young, elderly men outperform elderly women on mental rotation[60] and spatial perception tasks[39]. For spatial visualisation tasks, the findings are variable and task-specific. A male advantage was reported by some[11,24] although equivalent male and female performance was observed by others including on the block design task[11] and figure copying[39], mirroring the conflicting patterns seen in the young.

MEMORY

Little evidence supports an unambiguous sex difference in memory function for the healthy elderly. Some report women being better at immediate word learning[47],

Table 1 Sex differences in studies assessing fluency and naming in the elderly

Ref.	Sample size		MMSE		Tasks	Finding
	M	F	M	F		
Marra *et al*[41]	85	168	19.1	17.6	CF (fu)	NS
					CF (b)	M > F
Monsch *et al*[42]	43	46			LF (F, A, S)	NS
					CF (s, *n*)	NS
					CF (a + f + ve)	F > M
Beinhoff *et al*[45]	26	23	25.6	24.7	CF (a)	NS
					CN	NS
Henderson *et al*[46]	22	24			CN	M > F
Henderson *et al*[46]	270	377	17.5	17.3	CF (a)	M > F
					CN	M > F
Moreno-Martinez *et al*[49]	28	33	21.2	18.9	CF (I, tr, v, t, mi)	M > F
					CF (a, fl, f, fu, k, c, bg, bp)	NS
Randolph *et al*[53]	129	196			CN	M > F
Buckwalter *et al*[55]	72	87	17.8	16.5	CN	M > F
McPherson *et al*[77]	23	36	23.3	22.2	LF (F, A, S)	NS
					CF (a)	NS
					CN	M > F
Ripich *et al*[78]	29	31			LF (F, A, S)	NS
					CN	M > F
Bayles *et al*[79]	30	33	15.2	15.9	LF (A, S)	NS
					CF (a, f)	NS
					CN	NS
Perneczky *et al*[81]	50	43	23.9	23.0	CF (a)	NS
					CN	M > F
Henderson *et al*[136]	26	27	13.8	11.8	LF (F, A, S)	NS
					CF (a)	NS
					CN	NS

F: Female; M: Male; NS: Not significant; MMSE: Mini mental state examination; CF: Category fluency; CN: Confrontation naming; LF: Lexical fluency; CF: Categories; a: Animals; b: Birds; bg: Buildings; bp: Body parts; c: Clothing; f: Fruit; fl: Flowers; fu: Furniture; i: Insects; k: Kitchen utensils; mi: Musical instruments; n: First names; s: Supermarket items; t: Tools, tr: Trees; v: Vehicles; ve: Vegetables.

verbal memory and episodic memory[22], while others have found no sex differences in verbal memory[61] or for delayed word learning[47,50]. Some evidence also documents that elderly men have better visual memory[38], working memory and episodic memory[24]. In summary, sex-differences in the memory ability of the healthy elderly where described, appear to be significantly dependent upon the specific task.

We have reviewed the evidence for sex and age-related differences in cognitive tasks within the normal population and principally within the healthy elderly population. It is important to have a clear understanding of such patterns, and the extent to which they are well-replicated, if we are to make sense of any sex differences in cognitive abilities that may emerge after the onset of AD.

VERBAL ABILITIES IN AD

Semantic memory impairments emerge early in AD neuropathology[62]. Category fluency deficits may be apparent as much as five years prior to diagnosis[63] and mild AD patients are more impaired than those with Mild Cognitive Impairment (MCI) and elderly controls on category fluency[64,65]. Two meta-analyses[66,67] have highlighted large significant category fluency deficits in people with AD compared to healthy elderly controls, with Laws *et al*[67] calculating an extremely large effect size in 92 studies (d = 2.10).

The pattern with regard to lexical fluency deficits, however, is more varied with some finding no impairment[68,69], but others a worsening of performance[70]. Nonetheless, meta-analytic studies[66,67] confirm a reliable AD deficit in lexical fluency, with[67] estimating a large effect size (d = 1.46).

Compared to the healthy elderly, AD patients also have far greater difficulties on confrontation naming tasks[64,69,71-75]. A meta-analysis of 56 naming studies assessing 2607 AD patients and 2285 healthy controls by Laws *et al*[67] obtained a large effect size deficit in AD patients (d = 1.54).

SEX DIFFERENCES IN VERBAL ABILITIES IN AD

While AD is characterised by decline in the verbal and semantic domains, does any evidence suggest that the relative performance of men and women differs from the pattern in the healthy elderly? A search of the literature reveals a modest number of relevant studies (Table 1).

Several studies in the 1990s reported that, compared to men with equivalent AD severity, women manifest a more profound impairment of semantic memory[46,76-78].

Table 2 Studies assessing sex differences in visuospatial abilities for Alzheimer's disease patients

Ref.	Sample size		MMSE		Tasks	Finding
	M	F	M	F		
Beinhoff *et al*[45]	26	23	25.6	24.7	FC	M > F
Henderson *et al*[46]	22	24	Not given		FC	NS
Henderson *et al*[46]	270	377	17.5	17.3	FC	NS
Buckwalter *et al*[55]	72	87	17.8	16.5	BD	NS
					FC	NS
Perneczky *et al*[81]	50	43	23.9	23.0	FC	NS
Cushman *et al*[90]	22	12	24.03		JLO	NS
Heun *et al*[101]	171	267	15.5	16.3	FC	M > F
Millet *et al*[102]	20	20			Corsi,	NS
					Corsi (b),	M > F
					VPT	
Henderson *et al*[136]	26	27	13.8	11.8	FC	NS

M: Male; F: Female; NS: Not significant; MMSE: Mini mental state examination; BD: Block design; Corsi: Corsi block tapping; Corsi (b): Corsi block tapping (backwards); FC: Figure copying; JLO: Judgment of line orientation; VPT: Vecchi's pathway task.

In particular, female AD patients name fewer items correctly in confrontation naming tasks[46,55,77,78]. Others have reported higher naming scores by men, but no significant sex differences[45,79]. Crucially, the significant sex differences in AD patients remain after controlling for the effects of age, education, and duration of illness[46,55,76,78]. In their meta-analysis, Laws *et al*[67] confirmed a male naming advantage by showing larger effect sizes in studies containing a greater proportion of female AD patients.

Several studies have reported no sex differences in lexical fluency performance in AD patients[42,77-80] but a more ambiguous profile has emerged with category fluency tasks and may reflect the specific choices of categories. Perhaps surprisingly, men with AD significantly out-perform women on the most commonly used category of animals[46] as well as for insects, trees, tools, musical instruments, vehicles[49] and birds[41]. Nevertheless, others have found no significant sex differences for: Animals[45,46,49,77,79,81], fruits[49,79], furniture[41,49] supermarket items or first names[42]. Only one study[42] has asserted a female semantic fluency advantage, though this was for the total of animals, fruits and vegetables combined.

VISUOSPATIAL ABILITIES IN AD

While verbal and memory problems in AD are widely acknowledged, the prevalence of visuospatial deficits has been relatively underplayed, and may be important given the link between visuospatial abilities and functional competence in healthy older individuals[82] as well as those with dementia[83]. Some evidence also intimates that visuospatial tests may be useful in staging the disease process, differentiating mild from moderate dementia in AD[84].

People with AD fare worse than the healthy elderly on visuospatial tasks, including, for example, tests of mental rotation[85-88]. Lineweaver *et al*[88] posited that since mental rotation involves the parietal cortex, and AD results in extensive damage to this region, AD patients would be impaired at this task. This was indeed the case when compared with healthy elderly controls[88].

Salmon *et al*[89] claimed that the visuospatial deficits associated with AD are usually evident in visuoconstructional tasks (*e.g.*, block design) and visuoperceptual tasks [*e.g.*, judgement of line orientation (JLO) task]. In line with this, several studies show that AD patients are worse than elderly controls on the JLO task[88,90-92], although Finton *et al's*[93] AD patients presented with no problems on this task. Impairments have been reported both for block design[91,94] and for figure copying[95,96]. Even participants with mild AD score lower than elderly controls on a figure-copying task[97,98] and on drawing a complex figure from memory[98].

Visuospatial deficits are also apparent for some years prior to AD diagnosis. Backman *et al*[99] found that healthy elderly individuals, who were later diagnosed with AD, performed worse on visuospatial tasks than those who remained free of dementia at follow-up. In a related vein, Laukka *et al*[100] identified an increase in the rate of visuospatial decline in elderly participants up to 10 years prior to AD diagnosis.

SEX DIFFERENCES IN VISUOSPATIAL ABILITIES IN AD

Although AD reduces performance on a range of visuospatial tasks, does the performance on these tasks differ between men and women? If AD affected men and women equivalently, then we might expect to see a continuing male advantage on most visuospatial tasks, as an extension of the typical healthy elderly profile.

Of 16 studies that have considered sex differences in AD patient cognition, nine included at least one visuospatial ability task (Table 2). Only one used a spatial perception task[90], which was primarily concerned with navigation (they also included the JLO task) and no significant sex differences emerged. All other papers examined spatial visualisation tasks where, as already discussed, findings in the general population are variable and seem to be contingent on the specific task used. Buckwalter *et al*[55] found no differences between male and female AD patients on block design. Beinhoff *et al*[45], however, reported that AD males outperformed AD females at a drawing task measuring visuospatial episodic memory. These findings were unlikely to reflect a generalised visuospatial skill advantage as no sex differences emerged for visuospatial memory span. Most other researchers, however, have failed to discern any difference between AD men and AD women at copying a geometric figure[46,55,80,81,101]. Heun *et al*[101] did report a male superiority on visuoconstructive tests that involved copying geometrical figures such as cubes, but this did not reach significance.

In summary, the visuospatial abilities of men and women with AD do not parallel the male-female divergence seen in the healthy population. To date however, no paper has examined mental rotation performance in AD patients, which as discussed earlier, is the most sensitive visuospatial task to sex differences in the general population. Given the complexity of rotation tasks, researchers may feel they are less useful with AD patients. As noted, Lineweaver *et al*[88] did use a simplified rotation task, but they did not report male and female performance separately.

By contrast, some studies suggest that sex-based cognitive differences may disappear or even reverse in AD. For example, Perneczky *et al*[81] reported no significant sex differences in patients with mild AD on either verbal or visuospatial tests. One possible interpretation is that a proportionally greater deterioration of verbal and visuospatial ability occurs, respectively, for women and men. However, some contend that the male visuospatial advantage remains in AD sufferers, possibly on tasks that tasks requiring active manipulation of visuospatial information[102]. And Beinhoff *et al*[45] reported that AD males were superior at learning and retaining visuospatial information, though no sex differences in visuospatial memory span emerged. Turning to verbal abilities, Chapman *et al*[103] found greater accuracy in AD men on the Logical Memory test, which assesses verbal episodic memory, and this was a reversal of the profile in their healthy elderly controls. Surprisingly perhaps, male AD patients are also superior on naming tasks[46,55,77], and verbal fluency[55]. It is evident then that findings relating to sex differences in AD patient cognition are somewhat inconsistent, and the results of individual studies may even be misleading on this issue. Moreover, a failure to find significant sex differences in some studies may reflect insufficient statistical power; and thus, meta-analysis is a useful approach in this area.

Surprisingly few studies present neurocognitive data separately on males and females with AD. Irvine *et al*[104] identified just 15 published studies presenting data from a total of 828 men and 1238 women with AD. Unsurprisingly, AD studies contained more female than male patients (60% *vs* 40%) and although most researchers test both male and female patients, they do not routinely report between-sex comparisons, and so any differences have gone unnoticed. In earlier work, we tried to circumvent this methodological drawback by assessing the "proportion" of male participants per study as a moderator of effect sizes. This approach showed that picture-naming effect sizes increase in AD patients as the proportion of female patients increase[67]. Similarly, our meta-analysis[67] of 92 studies examining semantic and 96 examining phonemic fluency in over 4500 and 3000 AD patients respectively, found that the proportion of females significantly predicted both effect sizes. In other words, as most studies of AD patients have more females, studies will tend to significantly inflate effects, and differences in the proportions of male and female participants will increase variability in findings across studies.

Irvine et al[104] *meta-analysis*

The meta-analysis by Irvine *et al*[104] uncovered small, but significant male advantages across each of five cognitive domains examined (Cohen's *d*, 95%CIs, Table 3): Episodic memory (d = - 0.17, 95%CI: -0.33 to -0.01) semantic memory (d = -0.25, 95%CI: -0.42 to -0.07) verbal (d = -0.27, 95%CI: -0.37 to -0.16), non-semantic (d = -0.14, 95%CI: -0.26 to -0.02) and visuospatial (d = -0.24, 95%CI: -0.43 to -0.05). In terms of consistency, 49 of 52 (94%) effect sizes calculated by Irvine *et al*[104] were in the direction of worse female performance across varied cognitive domains. Furthermore, moderator regression analyses revealed that these deficits were not predicted by differences in age, education or overall dementia severity (as measured by MMSE). Hence, the worse cognitive performance of women with AD is not attributable to obvious demographic confounds.

What are the possible reasons for AD affecting the cognitive abilities in women more than in men?

One reason for the more pronounced decline in women might relate to men having greater cognitive reserve. Cognitive reserve has been defined as the amount of brain damage an individual can tolerate before reaching a clinical threshold for impairment[105]. Individuals with greater reserve are hypothesized to sustain more AD-related neuronal damage before onset of symptoms and clinical diagnosis. Consistent with this hypothesis, several recent neuroimaging studies have reported differences in brain function for male and female AD patients who are at the same disease stage. In accord with the greater age related cognitive decline in men, corresponding brain imaging evidence points to greater age-related brain deterioration in males than females[36,106]. Magnetic resonance imaging has detected greater age-related brain atrophy (as indicated by increased cerebrospinal fluid volume) in males than females[108]. In terms of specific regions and structures, greater age-related frontal and temporal lobe volume reductions have been described in males[109], while others[110] have reported a more specific reduction in hippocampal volume across early adulthood in males but not in females. A more recent and novel study using diffusion tensor imaging[111] assessed patterns of white matter connectivity - the connectome - in a large sample of males (n = 428) and females (n = 521) aged from 8 to 22, finding that females displayed stronger interhemispheric connections, while intrahemispheric connections seemed stronger in males. Although the study found no age-by-sex interaction, suggesting no sex differences in the developmental trajectory of connectivity, the duration covered was relatively limited.

In their post mortem analyses of 141 brains from the religious orders study, Barnes *et al*[21] found the association between AD pathology and clinical AD was significantly more likely to be expressed in women than

Table 3 Cohen's *d* effect sizes (95%CI) in different cognitive domains

Ref.	Sample size			Semantic	Non-semantic	Verbal	Visual-spatial	Memory
	M	F	Total	*d*	*d*	*d*	*d*	*d*
Marra *et al*[41]	85	168	253	-0.23		-0.23		
Beinhoff *et al*[45]	26	23	49	-0.07	-0.44	-0.22	-0.60	-0.37
Hendersen *et al*[46]	22	24	46	-0.37	-0.37	-0.37	-0.18	
Hendersen *et al*[46]	270	377	647	-0.30	-0.12	-0.30	-0.11	-0.12
Moreno-Martinez *et al*[50]	28	33	61	-0.42		-0.42		
Buckwalter *et al*[55]	72	87	159	-0.46	-0.24	-0.46	-0.24	
McPherson *et al*[71]	23	36	59	-0.24	-0.54	-0.35		-0.71
Ripich *et al*[78]	29	31	60	-0.74		-0.74		
Bayles *et al*[79]	30	33	63	-0.10		-0.10		
Perneczky *et al*[81]	50	43	93	-0.24	-0.12	-0.20	0.02	-0.17
Heun *et al*[101]	17	76	93		-0.18		-0.62	-0.04
Millet *et al*[102]	20	20	40		-0.40	0.08	-0.63	-0.40
Laiacona *et al*[107]	11	15	26	-0.29		-0.29		
Hendersen *et al*[136]	26	27	53	-0.26	-0.22	-0.09	-0.44	-0.15
Hebert *et al*[137]	119	245	364	-0.23	0.04	-0.09		
Total	828	1238	2066	-0.25 (-0.42 to -0.07)	-0.14 (-0.26 to -0.02)	-0.27 (-0.37 to -0.16)	-0.24 (-0.43 to -0.05)	-0.17 (-0.33 to 0.01)

Negative effect sizes favour men and positive effect sizes favour women; numbers in parenthesis are 95%CIs. M: Male; F: Female.

in men. Indeed, each unit of AD pathology (based on neuritic plaques, diffuse plaques, and neurofibrillary tangles in areas sampled from four cortical regions) increased the odds of clinical AD by more than 20 times in women compared with a 3-fold increase in men. Furthermore, with each additional unit of AD pathology, the cognitive function scores for episodic memory, semantic memory, working memory, perceptual speed, and visuospatial ability were significantly more reduced in women than in men.

Although male and female AD patients show some commonalities in functional imaging studies, differences in regional cerebral blood flow (rCBF) emerge, with women showing a more severe decrease of rCBF in the medial temporal region and frontal lobe[112]. Neuroimaging studies reporting sex differences in brain function for males and females at the same stage of the disease are consistent with a reserve hypothesis. Perneczky *et al*[81,113] found that despite being at the same disease stage; and showing no significant cognitive differences, men with AD had more pronounced and extensive pathology affecting the frontal, temporal and insular cortex, as well as the hippocampus in the right hemisphere. Moreover, women were more likely to clinically express reductions of regional cerebral metabolic rate as dementia. The authors suggest that this could be because the brain reserve capacity serves as a stronger counterweight to neurodegeneration in men than in women.

Early neuropathological progression appears to be independent of sex, but female MCI patients show an increased vulnerability to cognitive impairment earlier in the illness course; and women with AD show greater cognitive impairment than men, despite an apparent equivalence in brain atrophy[114]. Perneczky *et al*[113] reported more pronounced and extensive AD pathology for men than women in the frontal, temporal and insular cortices as well as the right hippocampus, despite being at the same disease stage and showing no significant differences in general cognitive abilities.

The apolipoprotein E (APOE) ε4 allele is an established genetic risk factor for AD[115]. Although estimates vary somewhat across studies and ethnicity, the *APOE ε4* allele is present in > 50% of AD patients and approximately only 15% of healthy elderly controls[116]. Crucially, *APOE ε4* affects the probability of developing AD more in women than men[117-120]. This common polymorphism increases the risk of clinical conversion more in women than in men both in the conversion from healthy aging to MCI/AD and in the conversion from MCI to AD[120]. Lin *et al*[121] have recently examined longitudinal rates of change over eight years in a large sample of 398 MCI subjects (141 females and 257 males), finding faster rates of cognitive and functional decline in women than men and this effect was greater in female *APOE ε4* carriers. In the healthy population, the impact of APOE *ε4* on cognitive performance is more pronounced in women[122,123], and has been specifically linked to hippocampal atrophy in female MCI sufferers[124]. A large post mortem study (n = 729) established that AD-related abnormalities such as neurofibrillary tangles and senile plaques are affected by a complex interaction between the aging process, sex, and genetic (*APOE ε4*) risk factors[125]. These findings are consistent with a relatively greater semantic and verbal impairment in female AD sufferers that differs from, and is greater than, any pre-existing sex differences in cognition[101].

Estrogen has been implicated in the pathobiology of AD[126]. Indeed, findings suggest that verbal sex differences in AD may arise *via* an estrogen deficiency in women. Further evidence shows that Estrogen therapy prevents the decrease in verbal memory when administered immediately following the surgical

removal of both ovaries in premenopausal women[127]. Women with AD who receive estrogen hormonal therapy perform as well on naming and verbal short-term memory tasks as men and significantly better than AD women not receiving such therapy. Further evidence suggests that duration of estrogen use is related to the rate of global cognitive decline and visuospatial ability in non-demented elderly women, although not to semantic or episodic memory[37]. Loss of estrogen alone cannot fully explain the poorer cognitive performance of women with AD; otherwise we would expect the same deficits seen in women with AD (for verbal fluency and verbal episodic memory) to be evident in the healthy elderly and this is not the case.

Following the menopause, cognitive abilities in healthy elderly women may be adversely affected by estrogen loss, albeit primarily on verbal tasks[127-130]. Indeed, women show significant changes in cognitive function during pregnancy and the postpartum period, principally in verbal free-recall and working memory[131,132], word fluency and word list learning[133]. Recent evidence suggests that during the third trimester and the early postpartum period, verbal recall deteriorates in pregnant women[134]. Furthermore, a longitudinal study of pregnant women showed they performed worse than non-pregnant controls on two tests of verbal memory, a visuospatial task, and on a task of processing speed[135]. These findings support the view that changes in sex hormone production within the physiological range that occur during reproductive events modify performance on a variety of cognitive functions - but principally on verbal tasks.

CONCLUSION

Although not unanimous, the evidence presented in this review converges on the multiple cognitive abilities being more adversely affected by AD in women than in men. This conclusion is strengthened by our own recent meta-analyses consistently affirming that men with AD outperform women with AD across a range of cognitive domains.

The literature on verbal abilities in the elderly reveals either an advantage for women or no sex difference - crucially, not one paper documents a male advantage in this domain. Findings are somewhat inconsistent in studies of cognitive decline under normal aging, suggesting something specific about AD neuropathology that disadvantages females. Some limited evidence suggests that females deteriorate faster than males in the earlier stages of the disease. Possible explanations are for a hormonal influence, possibly due to estrogen loss in women or a greater cognitive reserve in males, which provides protection against the disease process. Future studies which examine sex differences on a longitudinal basis, may provide greater clarity on these issues.

The unequivocal finding from the Irvine *et al*[104] meta-analysis of AD patients is that men modestly but significantly outperform women in all of the five cognitive domains assessed. Moreover, most papers report better male performance within every domain (only three had a female superiority in any single domain and the effect sizes were close to zero). Neither any differences in age nor dementia severity (as measured by MMSE) could account for the male advantage. Overall, the findings indicate that in women with AD, multiple cognitive functions are affected both more severely and more widely than men.

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REVIEW

Systems psychopharmacology: A network approach to developing novel therapies

Peter J Gebicke-Haerter

Peter J Gebicke-Haerter, Central Institute of Mental Health, Medical Faculty Mannheim, Heidelberg University, 68159 Mannheim, Germany

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Correspondence to: Dr. Peter J Gebicke-Haerter, Professor of Pharmacology and Toxicology, Central Institute of Mental Health, Medical Faculty Mannheim, Heidelberg University, J5, 68159 Mannheim, Germany. peter.gebicke@zi-mannheim.de
Telephone: +49-621-17036252
Fax: +49-621-17036255

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Abstract

The multifactorial origin of most chronic disorders of the brain, including schizophrenia, has been well accepted. Consequently, pharmacotherapy would require multi-targeted strategies. This contrasts to the majority of drug therapies used until now, addressing more or less specifically only one target molecule. Nevertheless, quite some searches for multiple molecular targets specific for mental disorders have been undertaken. For example, genome-wide association studies have been conducted to discover new target genes of disease. Unfortunately, these attempts have not fulfilled the great hopes they have started with. Polypharmacology and network pharmacology approaches of drug treatment endeavor to abandon the one-drug one-target thinking. To this end, most approaches set out to investigate network topologies searching for modules, endowed with "important" nodes, such as "hubs" or "bottlenecks", encompassing features of disease networks, and being useful as tentative targets of drug therapies. This kind of research appears to be very promising. However, blocking or inhibiting "important" targets may easily result in destruction of network integrity. Therefore, it is suggested here to study functions of nodes with lower centrality for more subtle impact on network behavior. Targeting multiple nodes with low impact on network integrity by drugs with multiple activities ("dirty drugs") or by several drugs, simultaneously, avoids to disrupt network integrity and may reset deviant dynamics of disease. Natural products typically display multi target functions and therefore could help to identify useful biological targets. Hence, future efforts should consider to combine drug-target networks with target-disease networks using mathematical (graph theoretical) tools, which could help to develop new therapeutic strategies in long-term psychiatric disorders.

Key words: Polypharmacology; Drug-target networks; Network topology; Disease networks; Genome-wide association studies; Epigenetics; Schizophrenia; Herbal extracts

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Core tip: Pharmacotherapies of mental disorders have to take into account their multifactorial origins. Therefore, combinatorial drug therapies have to be devised

targeting multiple molecules in disease networks. Mathematical approaches can aid in learning more about molecular interactions in disease networks and, by in silico simulations, discover dynamic changes in network properties. Targeting important nodes by drugs should be avoided to preserve network integrities. Efforts to find efficient combinations of drugs could be supported by more research into compounds contained in herbal extracts.

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INTRODUCTION

It is widely accepted that many diseases, in particular disorders of the Central Nervous System are multifactorial of origin. Consequently, reasonable pharmacotherapies should aim at addressing those multiple factors causing and sustaining the diseases, simultaneously. However, only recently strategies of "polypharmacology" (one drug adressing several targets) have appeared in research and in the literature. Apparently, the drug industry has relied for a long time on a small number of targets that had already been validated, subsequently generating an abundance of "follow-on" drugs. Statistical analyses of large component size, degree distribution and clustering coefficient quantitatively confirm this bias. This bias has been reinforced by the conviction, that the "major" targets known for most mental disorders were sufficient for the development of efficient pharmacotherapies. However, with the advent of modern sequencing technologies facilitating the sequencing of whole genomes within reasonable times and declining costs, intensive efforts to search for more targets, preferably on the gene level, have gained new steam.

GENOME-WIDE ASSOCIATION STUDIES

The idea behind genome-wide association studies (GWAS) was to identify all genetic changes eventually causing a disease. Until very recently, however, browsings of whole genomes for sites of associations with a mental disorder have not met with the expectations, because they did not stand up to rigorous statistical analysis or were not sufficiently specific for only one mental illness[1]. Moreover, it has to be mentioned, that these tremendous efforts have been burdened by the fact, that an estimated 99% of all single nucleotide polymorphisms (SNPs) are benign and without adverse effects. Nevertheless, it has to be acknowledged that these are unbiased approaches with the potential to discover new pharmacological targets.

Many so-called "candidates" have surfaced in schizophrenia (SCZ) research (such as COMT, NRG, dysbindin), but for instance in a larger European ancestry sample, where 14 candidate genes had been detected[2], associations were equivocal. In another more recent study on psychosis, the authors came to the conclusion, that "No individual SNP showed compelling evidence for association with psychosis"[3]. Furthermore, in a recent study on anorexia nervosa (AN) encompassing 5551 AN cases and 21080 controls, there were "No findings that reached genome-wide significance", concluding that the sample, the largest yet reported for this disorder, was underpowered for their detection[4]. Apparently, these statistical hurdles were overcome by the efforts of the Psychiatric Genetics Consortium, resulting in the identification of 22 loci significant for association to SCZ, and considered to be statistically sufficiently robust[5]. It was estimated that more than 8000 SNPs independently contribute to SCZ, which confirms that SCZ is a highly polygenic disorder[6]. A subsequent analysis by the same consortium encompassing very large samples (approximately 37000 cases and 113000 controls) revealed 128 independent associations spanning 108 defined loci (including at least 600 genes) that are genome-wide significant for SCZ[7].

In an attempt to assign a physiological meaning to some genes belonging to those loci, genes linked to voltage-gated calcium channel subunits (*CACNA1C*, *CACNB2*, and *CACNA1I*), involved in glutamatergic neurotransmission and synaptic plasticity (*GRM3*, *GRIN2A*, *SRR*, *GRIA1*) and the dopamine D2 receptor gene (*DRD2*), "the target of all effective anti-psychotic drugs" have been discussed. Apart from doubts raised recently that the symptoms of psychosis or SCZ are caused by the over activity of dopamine[8], hypothesis-free investigations should rather serve to broaden our view of the disorder by searching for additional genes of interest not belonging to the main stream of thinking. Dopamine and glutamate neurotransmission appear to be involved in too many essential brain functions as to be suitable for drug therapies specific for SCZ. Another very popular category of genes identified in the GWAS loci were genes with important functions in the immune system, nourishing the belief brought up in quite some publications before, that SCZ is an autoimmune disorder[9]. However, the data published in this respect are weak and controversial, so that repetition of this hypothesis does not render it more likely. Moreover, a recently published investigation on SCZ and multiple sclerosis (MS) samples[10], focusing exactly on risk alleles of the HLA group of genes, reports on associations of these genes with both SCZ and MS, but with an opposite directionality of effect of associated HLA alleles (that is, MS risk alleles were associated with decreased SCZ risk). Our studies may also be in this line of results, revealing that many more genes of the immune system were downregulated in post-mortem brains of SCZ patients, than upregulated. However, more importantly, as pointed out in our paper, many of

those gene products may have functions in the Central Nervous System distinct from their immunological functions[11]. This notion had already been raised before by other groups who discovered the involvement of *MHC* genes in signal transduction of glutamate receptors and synaptic plasticity[12,13], which attributed a feature to MHC distinct from its immune function in the aetiology of SCZ[14].

Since all these recent studies required enormous logistic coordination and substantial financial support, they deserve to be scrutinized for their cost-benefit ratio in more detail.

As already alluded to above, associations have been found with genomic regions ("loci"), not with single genes. Genomic loci typically encompass more than one gene, so that it remains elusive, which gene is affected. Moreover, a SNP may or may not influence expression of genes within - or outside - the locus. And SNPs may or may not interfere with non-protein-coding genes (or unknown genes), or with annotated protein-coding genes[15] .

Very unlikely the identified SNP is the causative SNP, but only tags the stretch of DNA where the causal variant is located. Furthermore, all the SCZ -associated SNPs show up in non-coding regions of DNA (*e.g.*, intergenic, or intronic) or are synonymous exonic polymorphisms[16]. And above all, it has been underscored in a recent publication, that within approximately 7300 GWAS associations to common diseases or traits, only 20 could be clearly attributed to a causal variant[17].

Typically (and expectedly) the odds ratios associated with each SCZ risk SNP are around 1.10, indicating a very small effect on disease risk.

Some of the SNPs are not specific for the disorder, but also show associations *e.g.* with bipolar disorder or, to a lesser extent, with major depression, with attention-deficit hyperactivity disorder, and with autism (genetic pleiotropy)[18,19]. Hence, the clinical overlap between these disorders may arise to some extent from a shared genetic predisposition.

It is well known that SCZ is a broad spectrum psychiatric disorder which poses substantial problems of reliabilities of inclusion/exclusion criteria of patients according to diagnostic manuals ICD-10 or DSM IV, manuals that have been repeatedly critisized and are in the course of being thoroughly revisited[20-22]. In this light, the extensive increase of *n*-numbers in GWAS is bound to increase the probability of irreproducibility of results. Therefore, it remains at least controversial, to what extent further increases in sample sizes will improve our understanding of the disorder[23,24].

It is still surprising that even from apparently robust "candidate gene" approaches of the pre-GWAS era[25,26], almost all GWAS studies, including Ripke *et al*[5] were unable to replicate any of those genes. For these reasons, it is still a matter of debate to what extent genome-wide statistical significance can be reliably used to decide which gene is a risk gene or not[27].

Only a minor part of the heritability of SCZ can be explained by the existing results[28]. As concerns the major part, one assumption implies, that rather than simply arising from cumulative effects of multiple independent genes, gene-gene interactions (epistasis) may have higher impacts on the genetic risk to develop the disorder[29].

There are preliminary clinical[29,30], and experimental data[31,32] supporting a role for epistasis in SCZ. Unfortunately, systematic investigations on epistatic events in SCZ are not available, and GWAS studies do not help that matter[33].

Conversely, the majority of genetic heritability cannot be explained by GWAS studies, neither individually nor collectively[34,35]. And for most of the associated variants no functional links have been provided[36].

Therefore, GWAS results are of limited value for closer insights into molecular mechanisms of the identified loci/genes, and far away from applications in translational medicine or pharmacotherapeutic interventions. A very important, additional point to be made here is that all those studies depend on statistical significant results, which require prohibitively high numbers of samples due to multiple testing and other statistical problems, which in turn increase the "noise" level churning up the spiral more and more. On the other hand, gene modifications that do not result in any statistical significances because of their subtle effects, may synergize metabolically on the gene product level with each other or with additional genes not modified to exert significant contributions to the development of the disorder, meaning that not only genes reaching statistical significance are important. Very likely, there is much more passing undetected in the "noise" (see below, summary). Along these lines, it has to be assumed, that variation anywhere in the genome affects every character. In keeping with this, the notion was put forward, that there are "no special genes for psychosis". Instead, the "normal gene" model proposes that any gene or allele that influences the development of the human brain can tentatively act as vulnerability gene or allele, as well[37].

The disappointing failure to identify even one etiological candidate gene during many years of genetic studies on psychosis may be explained by the possibility that genetic vulnerability to psychosis is due to random mutations. Therefore, given the hypothesis that gene alleles that are endowed with general functions in brain development could also act as vulnerability gene alleles, it appears plausible that there is no need to postulate the existence of specific "psychosis genes or polymorphisms".

FROM GENETICS TO EPIGENETICS

Moreover, epigenetic modifications of various susceptibility genes with minor effects may well reinforce the development of psychosis[38]. Possibly, the combined effects of those genes along with their interaction with environmental factors results in a number of distinct

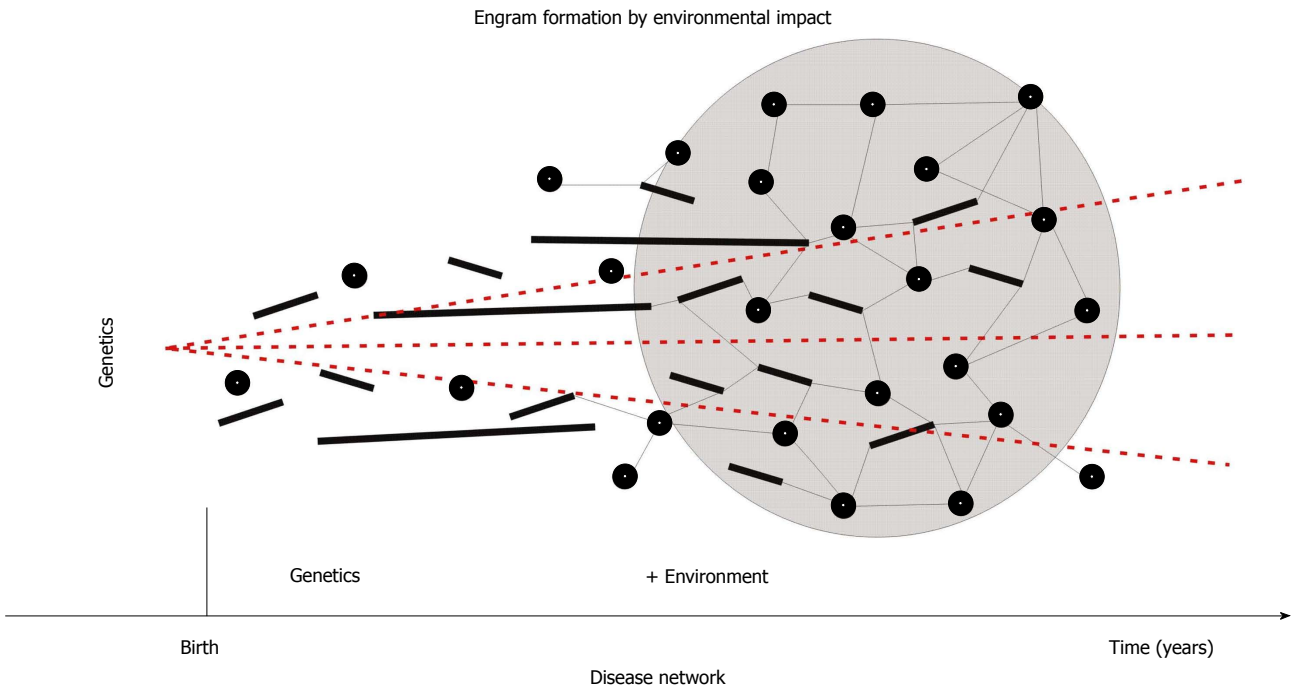

Figure 1 Theoretical formation of a disease network in human brain. Short-term (*e.g.*, traumatic = circles), medium-term (short bars), and long-term (*e.g.*, family = long bars) adverse impact emboss molecular engrams that eventually synergize to form a disease module or network (as shown by connections between nodes). Because the development of the human brain occurs predominantly postnatally, these environmental (epigenetic) influences appear to have more importance than the underlying genetic vulnerability (dashed lines).

phenotypes. In other words, a wide array of genes is working through various intermediate developmental and physiological pathways on different molecular levels. Changes of gene expression impact on protein expression, which interferes with cell metabolism (*e.g.*, in neurons)[39]. However, often directionality is reversed feeding back from "higher" levels down to ongoing activities at lower levels - thus the interactions are bidirectional[37]. This would account, at least in part, for the substantial heterogeneity repeatedly observed in the psychotic phenotype. As a matter of fact, there is strong evidence for environmental interference with regulation of neurodevelopment by means of epigenetic modifications. What is more, it has to be recalled that almost 80% of human brain growth falls in the postnatal period of life - this includes axonal growth, arborisation of dendrites, synapse formation, and myelination of axons. Therefore, this life span provides ample room for adaptation to environmental conditions and to organize brain development on the epigenetic level[40]. Apparently, the complex construction of brain, mind, and consciousness to enable the organism to respond adequately in a world of social interactions can better be optimized after the individual is born. Needless to mention, that the development of human intelligence and many more typical human characteristics (writing, language, abstraction, anticipation, *etc.*) are subject to environmental impact. Therefore, it comes of no surprise, that also the majority of psychiatric disorders arise primarily through problems of social functioning, social navigation, or social understanding. It appears safe to say that psychosis is not just a gene-driven by-product of brain evolution. Eventually, each society with its inherent adverse conditions of life gives rise to its specific psychosomatic or brain disorders. In this way, psychosis is an example of "socially sensitive" diseases and reflects structural deficits inherent of human societies. But also "simple" traumatic events, such as lack of oxygen supply during delivery may leave behind a traumatic "imprint" or "engram" that may be accelerated and reinforced by multiple, additional adverse events in childhood and adolescence (short or long-lasting, such as the influence of the family) and eventually result in the formation of a molecular "disease module" or a neuronal "disease circuit" (Figure 1).

This reasoning does not deny the presence of a genetic vulnerability being modified or reshaped postnatally by environmental influences and contributing its burden to the progression of the disease. However, even if a defect (polymorphism, copy number variant, *etc.*) is detected in a gene of known function, the phenotypic impact of that defect can only be seen in the context of the functions of the gene product's interaction partners, *i.e.*, by its network context[41]. There is no question that the most immediate reactions on environmental stimuli are located on the cellular level. These reactions may change or modify cellular components or subcellular, molecular interconnectivities. They may not only impact on predisposed genetic abnormalities but influence transcription on the epigenetic level (DNA methylations and posttranslational modifications of histone proteins)[39,40] and post-transcriptional events,

such as editing of mRNAs or mRNA degradation by small interfering RNAs or micro (mi)RNAs[42]. The latter way of posttranscriptional regulation of gene expression has become very popular to experimentally silence single genes as an alternative to produce gene knock-out animals. In the context of this review, however, it appears to be even more interesting, because those short RNAs (21-25 nucleotides in length) typically are not specific for one mRNA, hence display multi-target functions[43]. Expression levels of several hundreds of mRNAs can be modified by one miRNA, which results in "fine-tuning" of target gene expression. There are several reports delineating the occurrence of altered miRNA expression profiles in psychiatric disorders[44,45]. For example, miRNAs 1202 and 135 turned out to be involved in major depression disorder (MDD), supporting their role in influencing higher brain functions[46,47]. Owing to this relatively new field of research, the molecular mechanisms leading to altered miRNA expression in those disorders are largely unknown. Until recently, experimental and/or computational methodologies capable of detecting accurately and with high resolution miRNA gene transcription start sites (TSSs) were not available, although efforts in this direction have been made several years ago[48,49]. The latter, however, lacked reliable accuracy in experimental techniques, or presented *in silico* algorithms providing low resolution/high false positive rate predictions and heuristics. The first algorithm that surpassed the barrier of 54% sensitivity and 64.5% precision in miRNA TSS identification of the earlier studies by achieving 93.6% sensitivity and 100% precision is MicroTSS[50]. MicroTSS can accurately identify miRNA TSSs in single nucleotide resolution[51]. The interconnection between miRGen v3.0 and other DIANA resources enables users to identify *in silico* as well as experimentally verified miRNA targets on lncRNAs with LncBase[52]. This is very promising progress in getting more insight into regulatory mechanisms of miRNA gene expression and their influence on target mRNAs. A great challenge will be to identify methylation patterns and posttranslational modifications of histones of these genes in health and disease. As a result, effects on protein expression and disturbances of molecular networks in brain disorders may be better understood. Along these lines, it would be important to know, to what extent the products of genes targeted by miRNAs belong to disease networks (see below). Pharmacological interventions on miRNA gene expression would then be reasonable strategies to tackle the problem of the multifactorial origin of chronic psychiatric disorders. Consequently, a key hypothesis is that various pathobiological processes interacting within complex networks are continuously embossing a disease phenotype[39]. Generally speaking, it can be concluded that reductionistic biology (concentrated on single molecules) will not provide insights into the workings of those interconnected networks nor result in improvements of therapeutical solutions.

MOLECULAR NETWORKS IN HEALTH AND DISEASE

There are quite some efforts to abandon reductionistic biology and pave the way for a broader understanding of the maintenance of health and the initiation and progress of disease. Models of oscillating molecular networks could play key functions in identifying weak, but crucial variations in molecular interactions characterizing disease processes. For example, genome-scale metabolic (GSM) networks[53] have been used to investigate metabolic interactions at the cellular level. Additionally, as an attempt to improve the work with GSMs by computational modelling, "Flux Balance Analysis" (FBA) has been elaborated. This constraint-based modelling approach, or constraint-based reconstruction and analysis method, characterizes and predicts aspects of an organism's metabolism[54]. As a matter of fact, efforts to integrate mRNA expression data into metabolic networks could be significantly improved using FBA as an analytical platform[55]. For example, when growth of mutant *E. coli* was simulated with FBA, 86% of the mutant phenotypes (*i.e.*, growth or no growth) were accurately predicted[56]. Current GSMs stand out by their large sizes and by a rich source of annotations, but can be applied only to model protein relationships and reactions. More detailed modelling would need more sophisticated resources, which are hard to recover within a drug industry setting. FBA uses rates of uptake of extracellular metabolites and their production as input. The most important challenge appears to reside in the unclear relationship between gene expression and reaction flux[57]. As an advantage, this strategy can be used without biochemical data of enzyme kinetics or concentrations of intracellular metabolites. However, modelling a system using GSM networks is restricted to conditions of a pseudo-steady state, assuming, for instance, that cell proliferation is constant. There is quite some disagreement that the pseudo-steady state assumption is valid. Exact reaction kinetics, which would better reflect *in vivo* activity[58], cannot be reconciled with this approach. Nevertheless, these mathematical tools to study network behaviour paved the way to describe intracellular molecular interactions. They could be further improved, if they were replenished by modules of protein-protein interactions (PPIs) and by the influence of modules of signal transduction pathways. Overall, studies in this area would greatly benefit from the development of a good metabolite resource[59].

Some time ago, the total number of protein interactions within the so-called human "interactome" has been estimated to fall in the range of 130000-650000 interactions[60,61]. The wide range of variance is due to the fact that only subsets of these interactions have been experimentally identified. Networks have not only been used to gain insight into disease mechanisms[62,63], but also to study comorbidities[64], and to analyze the

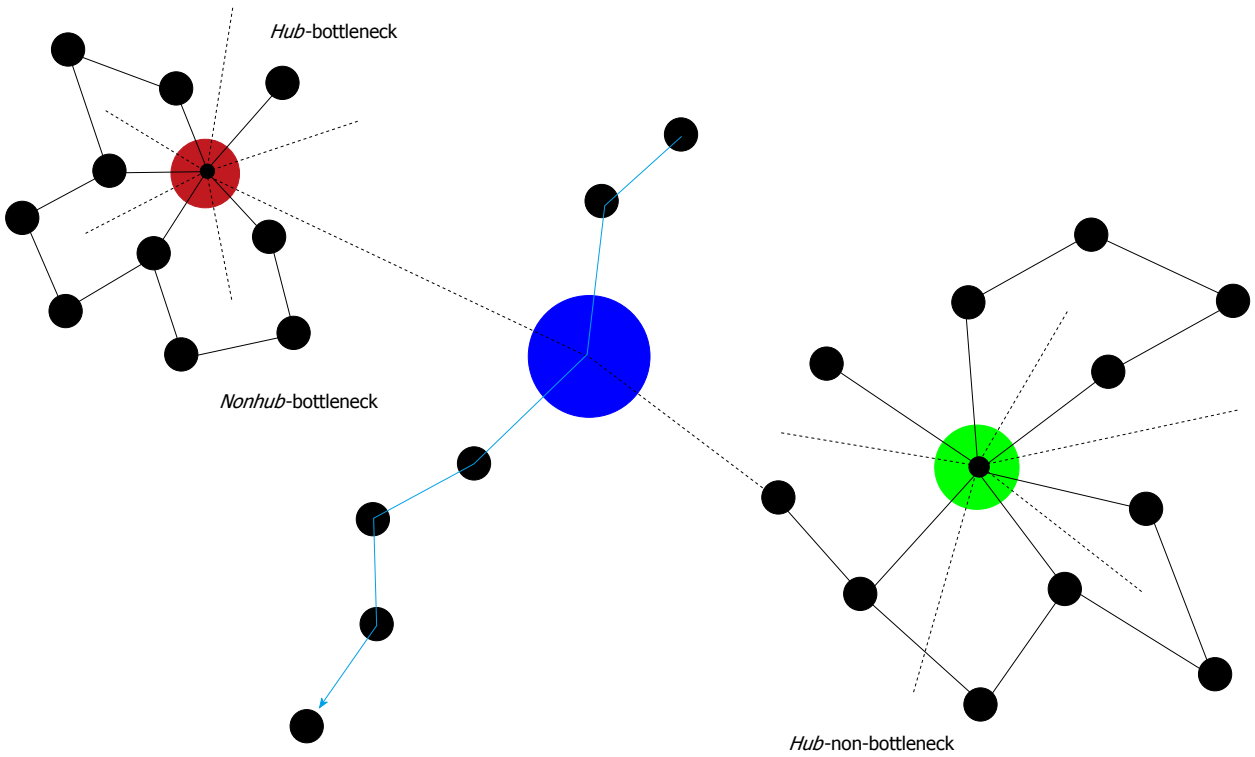

Figure 2 Some characteristics of protein-protein interaction networks. Hubs are important nodes (proteins) due to their numerous connections (dashed lines = connections in 3rd dimension). Directly inserted into a biochemical pathway, they can also represent a bottleneck (*Hub*-bottleneck). Otherwise, they are *Hub*-non-bottlenecks. *Nonhub*-bottlenecks are nodes inserted in biochemical pathways, but lacking numerous connections. Evidently, disturbances of any of these three important types of nodes result in serious consequences. Therefore, although associations of chronic mental illness with these types of nodes may exist, more unwanted side effects than benefits would ensue from targeting these types of nodes by pharmacotherapy.

actions of drugs and effects on their targets[65,66].

Some topological properties and mathematical tools to analyse those networks (Figure 2): Degree distribution in a network shows the number of connections of each single node, hence, identifies nodes with many connections as opposed to nodes with only few connections. In random networks, this distribution would look like a Gaussian curve of distribution, where few nodes have low numbers, and few have high numbers of connections whereas the majority of nodes have similar (mean) numbers of connections. Biological networks are different in that typically most of their nodes have very low connections (degrees), and with increasing connections there is a rapid dropoff of number of nodes with only very few nodes displaying high numbers of connections, obeying the mathematics of a power law distribution. For instance, the degrees of Scale-Free networks, as observed often in social networks, like Facebook, follow a power law, where a small number of nodes (people) are highly connected (hubs, see below). Hence, the low end of a power law distribution may indicate increasing importance within a network. However, the question arises here, if the importance of a node in a network is only dependent on its number of connections, which leads us to measures of centrality. A node with a high degree may occupy a central position within a network, or a peripheral position. Conversely, a node with a low degree (very few connections), may also be located in a central position. Hence, the question arises if it is possible to compute the centrality of a given node or how close any node is to any other node in a network. Closeness centrality is the most easiest way to determine it. It measures the average of the shortest pathlength of one node to every other node in the network. The result of this may be that nodes with low degrees may display higher closeness centrality, or display more importance, than nodes with higher degrees. Consequently, network structure and function may be affected more easily by attacks on these types of nodes compared to others with less closeness centrality. It may also give some hints as to the efficiency of transmission of information from those nodes to any other nodes of the network. The most widely used centrality measure is betweenness centrality[67], characterizing a node's influence. It is the percentage of shortest paths from every pair of nodes in the network. It gives us an idea what amount of information has to pass through each individual node. It has been proposed that edges located "between" highly connected subgraph clusters (so-called "community structures") are edges with high betweenness; consequently, a network could be disrupted by disabling these edges[68].

Eigenvector centrality is more sophisticated in adding weights to nodes (*e.g.*, ranking web pages) enabling identification of heavily used nodes *vs* nodes being used infrequently. Another important topological feature of networks is their clustering coefficient, which is an indication of neighbourhood connectivities.

In metabolic and other biological networks these connectivities are not random but some are favoured over others. Clustering algorithms are used to group sets of proteins in PPIs showing greater similarities among proteins of the same cluster than in different clusters which identifies functional protein modules or densely connected subgraphs. Several methods have been developed to search for such complexes. The Markov Cluster algorithm simulates a flow on the graph by calculating successive powers of the associated adjacency matrix[69]. Restricted Neighborhood Search Clustering is a cost-based local search algorithm calculated according to the numbers of intra-cluster and inter-cluster edges, that explores the solution space to minimize a cost function[70]. Molecular Complex detection is based on node weighting to isolate densely connected regions by local neighborhood density and outward traversal from a locally dense seed protein[71]. Spirin *et al*[72] developed a means to detect highly connected subgraphs (cliques) in combination with Monte Carlo optimization. The authors described two types of clusters: Protein complexes and dynamic functional modules. Furthermore, a highly connected subgraphs algorithm was used for discovery of protein complexes by Przulj *et al*[73], and spectral clustering for generating modules, and possible functional relationships among the members of the cluster for predicting new protein-protein connections has been proposed by Sen *et al*[74]. Along these lines, networks displaying small world characteristics should be mentioned. Compared to random networks, small world networks show intense local connectivities combined with more or less frequent large connectivities, displaying both low average shortest path lengths of random graphs and high clustering coefficients. The wiring of neuronal networks as well as of PPI networks shows features typical of small world graphs[75].

Hubs: A typical feature of hubs is their multi-connectedness or degree. Hub proteins appear to be encoded by essential genes[76]. These genes are older than genes encoding non-hub proteins and they are more stable over time[77]. Reportedly, products of essential genes are located in hub-like positions or, if expressed in multiple tissues, in functional centers of metabolic networks[78]. Due to this importance of hubs, the hypothesis arose that hub proteins in human molecular networks ought to be encoded by disease genes. However, it turned out later, that in human cells essential genes, but not disease genes, are encoding hubs.

Bottlenecks: Interestingly, protein clusters in interaction networks constructed by the method of edge betweenness show a strong tendency to display related functions[79]. These high-betweenness proteins were called bottlenecks[80] in contrast to hubs as proteins with high degree. Betweenness reflects the important role nodes would play in information transmission in the network. Proteins encoded by essential genes very often are positioned as bottlenecks (both nonhub-bottlenecks and hub-bottlenecks), whereas, surprisingly, proteins in positions of hub-nonbottlenecks are expressed by non-essential genes. The majority of proteins expressed by these genes are structural proteins, whereas proteins located in hub-bottlenecks rather belong to signal transduction pathways. A large part of proteins of nonhub-bottlenecks do not belong to complex members but to regulatory proteins or to proteins of the signal transduction machinery. Their coexpression with their neighbors in the networks is less well correlated than with proteins of nonhub-nonbottlenecks,, which is in agreement with the finding that betweenness is a good predictor of average correlation with neighbors. Apparently, inhibition or blockage of both hubs and bottleneck nodes may severely affect network integrity and function (Figure 2).

One critical feature to be considered in PPIs are post-translational modifications (PTM). More than 200 different types of PTMs have been identified, such as phosphorylations, glycosylations, methylations, acetylations, amidations, as detailed in http://www.uniprot.org/docs/ptmlist curated by UniProt[81] and other databases, like dbPTM, PTMCuration, PTMcode. For additional systematic searches of individual protein interactions, more databases, such as STRING[82], MINT and IntAct[83,84], for protein interactions within pathways, Wikipathways[85], Reactome[86] and Ingenuity (Ingenuity Pathway Analysis: http://www.ingenuity.com) are available. The volume of data held by the IntAct database alone, which includes just under 10% of the estimated human interactome[60], and currently encompassing a range of 50000 binary human interactions, may grow to 750000 binary interaction evidences in the next 5 years. More recently the issue was investigated if proteins exhibiting a particular type of PTM from a collected series of protein sets (displaying 12 types of PTMs) showed characteristic PPI network properties, such as scope of impact (interaction degree), diversity of responses (clustering coefficient), or position in a signalling pathway (closeness centrality)[87]. Interestingly, the 12 PTM-types could be grouped into 2 major groups with (1) sumoylation, nitrosylation, methylation, acetylation, phosphorylation, ubiquitination, and (2) disulfide bond, carboxylation, hydroxylation, proteolytic cleavage, glycosylation, and amidation. Not surprisingly, it turned out that there is a considerable overlap of PTMs, occurring in the same protein, especially with methylations, acetylations, and phosphorylations, indicating their joint associations with histones and their epigenetic functions. Results show that all PTM-types show a tendency of higher degrees, lower clustering coefficients, and higher closeness centralities than protein sets devoid of the respective PTMs. Furthermore, high degree proteins carrying the PTMs acetylation, phosphorylation, ubiquitination showed larger overlaps with human disease proteins than proteins with low degree.

Additionally, it is a challenge to involve the dynamic aspect into network studies by integrating complex data sets across time, space and different organizational

levels, providing a systems-level understanding. Therefore, in contrast or in extension to the above mentioned pseudo-steady state approaches, a recent review[88] welcomes various strategies to include the dynamics of biological networks and assumes that these approaches will be the de-facto network modelling in the future. As a matter of fact, the MINT-IntAct consortium is just beginning to implement this in their database, generating dynamic interaction data, in which dynamic changes in protein complex composition in response to stimuli are to be presented as animations driven by radio-buttons. All those mathematical tools and more sophisticated algorithms developed in the future will pave the way to analyze cellular (neuronal) and molecular (gene or protein) interaction networks in great detail, and identify modules or single nodes pivotal for their normal functioning. Moreover, simulations will provide insights into temporary profiles of those networks and detect sites of malfunction that may accumulate over time and set the stage of modules of disease, which reiterates to the model depicted in Figure 1.

DISEASE NETWORKS

Increased attention has been payed by the bioinformatics society to establish disease networks. These can be grouped into the following types of molecular networks: PPIs networks, whose nodes are proteins linked to each other *via* physical (binding) interactions; metabolic networks, whose nodes are metabolites that are linked if they participate in the same biochemical reactions; regulatory networks, or protein signalling networks whose directed links represent regulatory relationships, such as links between a transcription factor and a gene, or by other signalling molecules on downstream events or on PTM, such as those between a kinase and its substrates; and RNA networks, encompassing RNA-DNA interactions, such as small non-coding miRNAs and siRNAs in regulating gene expression.

PPI networks in particular have become very popular in this context[41]. PPIs entail binding characteristics between proteins and can also include PTMs and protein-protein dimerizations (see below).

Closer insights into the influences of molecular interconnectedness on disease progression could reveal gene products linked to disease and disease pathways, which, in turn, could offer more suitable targets for drug development. Additionally, these new targets could serve as better biomarkers that more accurately monitor the functional integrity of the network perturbed by the diseases. In this way, they could directly impact on clinical practice to achieve better classifications of disease and enabling earlier diagnosis and prognosis, which eventually aims at personalized therapies and treatment[20]. Therefore, analyses of disease networks are believed to permit a better understanding of the pathophysiology of chronic psychiatric diseases with the potential to design combinatorial pharmacotherapies[59].

Moreover, if infections have to be included in the consideration of disease progression, efforts have been undertaken to develop host-pathogen PPI networks[89]. These networks can lead to a better understanding of host-pathogen interactions and to identification of pivotal points for pharmacotherapeutic treatments. In all these approaches, the available databases have to be standardized to seamlessly enable sharing of data, such as being developed in BioPAX[90]. Along those lines, attempts were made to discover functional subnetworks tentatively related to the progression of colorectal cancer by combining analysis of mRNA expression with PPI data[91]. Here, a new computational algorithm was used to search for subnetworks embedded in a PPI network, that entailed genes differentially expressed in the disease. In another study, trying to elaborate predictions on metastasis in breast cancer, it has been shown that markers differentially expressed in subnetworks were more precise than single gene markers[92]. Hence, analysis of PPI networks is very useful to identify candidate biomarkers, to get more insights into disease mechanisms, and to obtain a better understanding of their biology. PPI network analysis also revealed significantly elevated protein interactions specific for the disorder. Two hundred and ninety of such interactions were identified, which corresponded to a 10-fold increase compared to random expectation ($P < 10^{-6}$)[93]. In other studies, similar results have been reported, *i.e.*, there are significantly increased, direct interactions of gene products associated with disorders of similar phenotypes[94,95]. From those observations, it can be concluded that once some disease components have been identified in the network, more disease-related components should be located in their neighbourhood. In other words, it seems likely that there are interactomes linked to diseases that are embedded in PPI-networks in well-circumscribed neighbourhoods. These interactomes frequently are named disease modules.

Along these lines, three distinct network modules should be considered (Figure 3): (1) topological modules; (2) functional modules; and (3) disease modules. (1) Topological modules stand out due to locally densely connected neighbourhoods of the interactome, *i.e.*, intramodular nodes preferentially interact within the module rather than with nodes outside of the module. In this respect, topological modules represent a pure network property; (2) Functional modules are distinguished for their significant segregation of nodes of related function (shown as circles in Figure 3, and connected by short dashed lines), and thus require to define some nodal characteristics. Their belonging to the same network neighbourhood is grounded on the assumption that intensities of nodal interactions are determined by their joint cellular functions; and (3) A disease module is a group of nodes showing changes (of expression, due to mutations, or epigenetic modifications) connected to a specific disease phenotype (drawn as squares in Figure 3, and connected by long, dashed lines).

The tacit assumption in network medicine is that the topological, functional, and disease modules partially

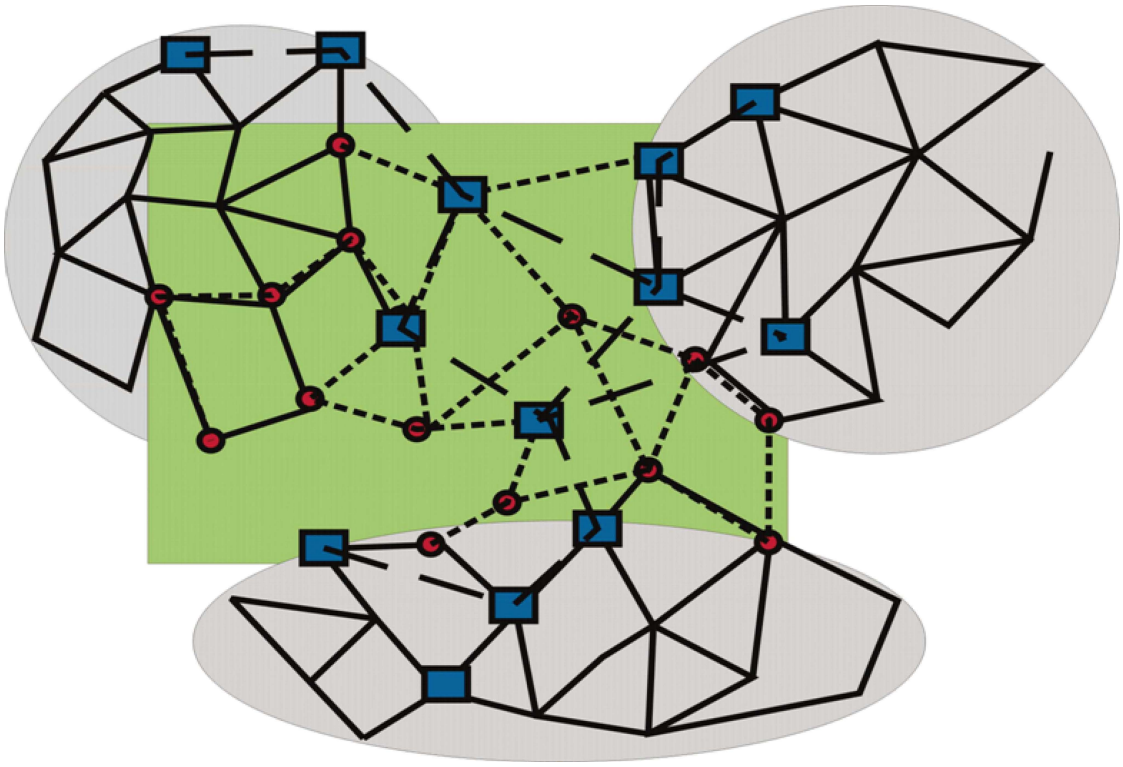

Figure 3 Topological, functional, and disease modules. Locally densely connected topological modules (grey circles) contrast with functional modules (circles connected by short, dashed lines), showing more (upper left), less or no overlap (center) with topological modules. The latter are preferentially associated with signal transduction pathways. Disease modules (nodes shown as dark squares, and connected by long, dashed lines) overlap with topological and functional modules, but may be less intensively connected and occupy more peripheral sites of networks.

overlap: Cellular components that form a topological module have closely related functions, thus being part of a functional module; and a disease is a result of disturbances in some functional module, which means that a functional module is also part of a disease module. However, several characteristics of disease modules are important to bear in mind. As pointed out, a disease module likely overlaps with the topological and/or functional modules, but, because a disease module is defined in relation to a particular disease, each disease has its own unique module. Optionally, a gene, protein, or metabolite can be implicated in several disease modules. There is general agreement in network medicine of mutual, partial overlaps of the topological, functional, and disease modules, which means that on the cellular level, topological modules typically are also closely related in their functions, hence being part of a functional module; and a disease results from disturbances in some functional module, which means that a functional module is also part of a disease module. However, it has to be pointed out, that albeit overlapping features of disease modules with the other modules, the definition of disease modules is specified by each particular disease, endowing these modules with some unique characteristics. Finally, when looking at single genes, proteins or metabolites in a particular disease module, it should be mentioned that each of these components can be a part of other disease modules.

A few years ago, PPI networks (disease networks) were constructed using data from genes differentially expressed in some psychiatric disorders[96]. The study revealed several disease markers (nodes or vortices) characteristic for SCZ (SBNO2), for bipolar disorder (SEC24C), and for MDD (SRRT). Furthermore, similar networks were constructed for Parkinson's disease (PD), using proteins differentially expressed only in substantia nigra and frontal cerebral cortex[97]. Construction of those networks was based on the following assumptions[96]: (1) There is a positive correlation between expression levels of most proteins and mRNAs in brain; (2) Proteins with similar expression patterns more likely interact with each other; and (3) The abundance of proteins correlates with their participation in biological processes.

In this way, thirty seven unreported disease marker genes were identified. Eight of them belonged to the core functional modules and four were strongly associated with some neurotransmitters, including dopamine. The results of this study may pave the way for addressing new targets in search for more efficient pharmacotherapy of PD. A more general study on the animal model of PD induced by the chemical MPTP used proteomics meta-data from the literature where neuronal alterations due to the metabolite of MPTP MPP+ had been reported[98]. The topological analysis of the protein networks generated on physical or functional interactions revealed a close interaction between nodes

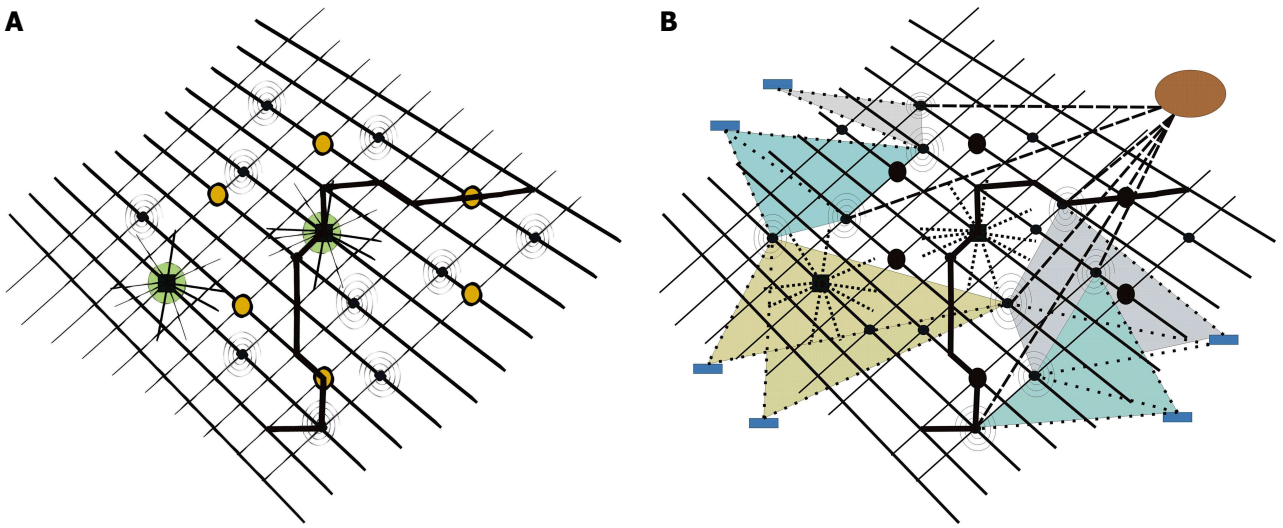

Figure 4 Molecular disease network. A: With two "hubs" (big, dark circles, showing high connectivity profiles) and six "bottlenecks" (dark circles), inserted in pathways (one pathway highlighted in bold), plus additional molecular nodes, tentatively disturbing network oscillations (spiral nodes, belonging to disease network); B: Subtle disturbances of network harmony by molecular nodes of minor importance (spiral nodes) may substantially interfere on a long-term scale in their summation with network oscillations and result in disease. Drugs with multi-target properties (circle upper right, strong, dashed lines), or multiple drugs with specificities for only one or a few targets (rectangles in periphery, weak, dashed lines) may address nodes of disease networks and reset disease networks to networks characteristic of healthy states.

as identified by an average shortest path length smaller than in random networks. Moreover, specific alterations in the mitochondrial proteome underlined in what way this model can recapitulate some pathogenic events of PD.

As mentioned above, the construction of those networks was based on the assumption that there is a positive correlation between expression levels of most proteins and mRNAs in brain. This may not be true in any case. Moreover, there are more steps between transcription of a gene and its product, such as post-transcriptional and-translational regulations, all of which complicate the correspondence between expression of a gene and its protein product.

DRUG-TARGET NETWORKS AND NETWORK PHARMACOLOGY

Traditionally, the focus in drug development is to interfere with the activity of one target molecule tentatively crucially involved in the onset or the progression of a disease. Typically, they evoke additional biological responses in patients, some of them leading to adverse or even toxic effects. Others may be benign. These benign effects may result from additional drug effects on additional targets beyond specificity ("dirty drug"). Research to identify those additional targets may open up new therapeutic options for the drug, which is well known in pharmacology as "drug repositioning"[99]. These basic principles constitute the basis of drug-target networks (DTN). Drug discovery can benefit from concepts of DTN in two ways: As polypharmacology and computational drug repositioning. Polypharmacology takes into account the above mentioned features of many drugs to be "promiscuous" in their specificities addressing more than one target molecule[100]. Specific changes of gene expression profiles in a cell are results of direct or indirect responses to a drug or a disease. Disease-induced disturbances of expression profiles could be conceived as perturbations of the dynamic, equilibrium state of a PPI. Drugs ideally may (re-) organize these molecular networks (drug-induced profile) and reverse disease-induced profiles into a state towards the dynamic, equilibrium state (Figure 4). Often, drug discovery is accompanied by investigating biochemical pathways. Because, however, in complex diseases multiple pathways may not be functioning in dynamic equilibrium, it may be advisable to use DTN to search for targets not necessarily being connected at the pathway level, but interacting more specifically on the disease level. Drugs expressing multiple target specificities with little adverse effects or displaying better tolerance have frequently been discovered in natural sources. In contrast to many currently approved drugs, natural products can be viewed as multi-component complex systems with therapeutic potential for a variety of diseases. They display many biological activities and good drug-like properties, show vast chemical diversity and can interact with multiple cellular target proteins[101]. Moreover, biologically active natural products are able to influence disease-related pathways, could provide selective ligands for disease-related targets[102], and could eventually shift the biological network from disease status to the healthy status.

One very well-known drug in this respect is acetyl-salicylic acid. Salicylates have been known for centuries as anti-inflammatory substances and were already used as extracts from the willow tree (*Salix Alba*) in the form of tea in times when these active substances

had not been identified. While the anti-inflammatory properties of salicylates are due to their inhibition of the NF-κB pathway[103], the serendipitous modification of salicylic acids by an acetyl group endows aspirin with a variety of additional characteristics. The substance inactivates cyclooxygenases through acetylation of serine residues[104]. Some 33 cellular proteins have been identified as acetylation targets of aspirin, one being the tumor suppressor protein, p53 at K382, inducing expression of its target genes[105]. Furthermore, enzymes of the glycolytic pathway (such as glyceraldehyde-3-phosphate dehydrogenase, enolase, aldolase, pyruvate kinase M2, and lactate dehydrogenase A and B chains), cytoskeletal proteins, histones, ribosomal and mitochondrial proteins are targets of aspirin modification. Aspirin also acetylates enzymes involved in ribonucleotide biosynthesis, such as glucose-6-phosphate dehydrogenase and transketolase[106]. Additionally, induction of apoptosis by activation of p38 kinases[107], and catabolism of polyamines[108] have been related to anti-cancer mechanisms of aspirin[109,110]. Regular intake of aspirin has been shown to reduce the risk of cancer of the esophagus by 73%, of the colon by 63%, of the stomach by 62%, of the breast and prostata by 39%, and of the lung by 36%[111]. The inhibition of G6PDH with increasing concentrations of aspirin is believed to be a crucial event in its anti-cancer effect, because this enzyme regulates the synthesis of nucleotides and nucleic acids. Along these lines, the activation of the ERK pathway mediated by the high levels of Ras mutations observed in many cancers[112], has been shown to be inhibited by aspirin, as well. Antiproliferative effects have also been reported from actions of salicylic acid, that is able to reduce mitochondrial calcium uptake[113]. Finally, aspirin has been shown to activate CREB, facilitating its binding to a cAMP-response element in the promoter of the neurotrophic factor CNTF and increasing its gene expression[114]. Aspirin (salicylates) is only one example of multi target effects exerted by many natural substances. Very likely, many other substances occurring in plants may be superior to synthetic drugs with high specificity for one target. For that reason, it may be more beneficial to learn from nature in what ways pharmacotherapies could become more efficient. Polypharmacology, indicating multi-target strategies in pharmacotherapies, encompasses those attempts to identify the multi-target nature of natural substances, but also to develop synthetic drugs with multiple target properties[115]. Especially in chronic brain disorders, these drugs may offer higher efficiency and less unwanted adverse effects[116]. In these terms, successful drug discovery in the pharmaceutical industry is still in its infancy, slowly trying to abandon the point of view that successful drugs should interact only with single, individual targets and approaching concepts of systems biology models of human metabolic networks[117]. Investigations on DTN on one hand, and disease networks on the other hand can aid to find overlapping targets and achieve better understanding of the mechanisms of action of multi-target (natural) compounds. The goal, hence, is to understand drug targets in the context of cellular and disease networks using systems pharmacological approaches[118].

Generally, hundreds of different biologically active substances are found in herbal extracts[119] and in contrast to most of synthetic drugs designed to bind single targets, most of the ingredients of herbal formulae display only weak to moderate effects, but address multiple cellular targets during treatment of complex diseases[120]. Normally, the underlying mechanisms are not clear. As a newly emerging field, network pharmacology[121] could help to understand the mechanisms of multiple action drugs across multiple scales from the molecular and cellular level to the tissue and organism level by analyzing the features of biological networks[122]. Network pharmacology is supposed to integrate polypharmacology and network biology and considered as upcoming paradigm in drug development[123].

It is exciting to study network pharmacology in product-target networks using natural products. Recent results on network properties suggested a marked enrichment of polypharmacology with respect to nodes (compounds) with large degree and high betweenness centrality. These nodes turned out to be highly influential in the whole network. Despite a slow change of direction in drug research and development, it has to be acknowledged that until recently approximately every second drug approved by the FDA was a natural product or a derivative thereof[124]. That means, that also in the industrial context increased efforts have been made to explore in more detail the mechanisms of action of herbal formulae employing network pharmacological approaches, such as DTN[125], PPI networks[126], metabolic networks[127], or disease networks (see above)[128]. In order to speed up virtual screening of natural products on a large scale, the Universal Natural Products Database has been constructed. Until now, it entails 197201 natural products (http://pkuxxj.pku.edu.cn/UNPD). Due to the complexity of studies using network pharmacology approaches, most of them are based on static networks[129]. However, network pharmacology also provides a systems-level approach to understanding the development and pathogenesis of disease, taking into account the dynamics of biological networks[130].

The mechanisms of action of natural products have been studied by well established, modern technologies, such as gene expression microarrays[131], technologies in proteomics[132], and metabolomics[133]. The concept of "network targets" emerging from these investigations extends the widely used concept of a drug tailored for a single biological component to the concept of a drug or group of drugs exerting multiple effects on a biological network[134]. The problem presently remaining is to develop mathematical algorithms able to configure herbal formulae holistically as a gestalt, whose emergent and tentatively synergistic properties no longer rely on analyzing each substance separately. Exemplarily, components of the Liu-Wei-Di-Huang pill

have been investigated completely *in silico* to predict their effects and mechanisms[135].

After analyzing the composition of chemical groups in LWDH, their chemical characteristics and distribution in chemical space were studied. Then their pharmacological properties were explored. Based on these results, predictions were made as to what biological molecules could be targeted by LWDH components. It was suggested that a biological molecule would be a "good" candidate target if there were several components specific for that biological molecule in the natural product. The results revealed that PPI networks constructed from the predicted candidate targets of LWDH displayed high intranet connectivity. Therefore, a compound-target network was constructed from the PPI network, where compounds were connected to their targets[136]. Then a disease network could be constructed by adding edges between candidate target proteins and a disease if the target protein's gene was in a gene list related to the disease. The results show that some target proteins belonging to hormone signalling, such as ESR1, NCOA1 and AR, are not only highly connected to the components of LWDH (high degree), attributing hub-like properties to them, but also highly connected to disease. However, a disease might be influenced by many biological processes that are targeted by different groups of ingredients, and hormone signalling is only one example. Like in many studies before, this study reveals, as well, that often "key players", here hubs and bottlenecks in molecular networks, become a focus of major interest.

CONCLUSION

There are numerous mathematical tools available to analyze networks both on the static and dynamic levels, and to distinguish structural components required for maintenance of their connectivities or responsible for their malfunctioning. Apparently, one major challenge for future studies is to improve algorithms taking into account changes of PTM over time and incorporate many more molecular nodes into these observations than just some "important" ones. The notion to be delineated here, is that efforts to identify "key" players in molecular networks may be misleading. Although they are often involved in maintenance and progression of a chronic mental disorder, they are also crucial for maintenance of many other metabolic functions independent of the disease. Therefore, interference by drugs with these crosspoints may easily destroy network integrity and, hence, be accompanied with numerous unwanted adverse effects. We rather want to conclude from the features of molecular networks outlined above, that it is more desirable to search for targets with more subtle effects on network functions, but specifically perturbed in a mental illness, and hence belonging to a disease network, as well (Figure 3). Along these lines, Lamb[137] have shown that mere node connectivity (degree) might not be the only influential parameter to characterize biological networks. And Goñi *et al*[138] reported that in case of neurodegenerative diseases, less extensively connected proteins are much more appropriate therapeutic targets than highly connected ones, as the critical role of highly connected nodes (hubs) in the network modules prevents them from substantial fluctuation. Exceptions probably are genes of miRNAs displaying "hub" features as well, that nevertheless may be good pharmacological targets. Moreover, it was shown that the above mentioned betweenness centrality can also be used as an important parameter to search for lowly connected nodes[80]. Chronic degenerative disorders of the brain extend over considerable periods of time, which strongly argues for disturbances of multiple nodes with weak influences, each. Or conversely, if abnormally functioning hubs or bottlenecks were the only players, the disorders would not be long-lasting and chronic. Consequently, in order to correct "damages" in disease networks, future pharmacological strategies to treat mental disorders may be aimed at targeting "peripheral" molecules with only subtle effects using polypharmacological approaches. The great challenge, hence, is to identify those "peripheral" targets and develop wide spectrum drugs aimed at those targets. In summary, it has to be kept in mind, that the human brain both in health and disease is a biological system distinguished by its extremely high complexity especially on the molecular level. Mathematical approaches to investigate changes on this level still require many improvements, and even greater challenges are confronted when it comes to address dynamic changes of the system. Because, however, this is at the core of biological systems, there is no way around. Reductionistic attempts to understand molecular mechanisms of mental illness are not able to address these issues adequately.

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REVIEW

Are the changes in the peripheral brain-derived neurotrophic factor levels due to platelet activation?

Montserrat Serra-Millàs

Montserrat Serra-Millàs, Osona Mental Health, Hospital Universitari de Vic, Salut Mental i Innovació Social (SaMIS), Universitat de Vic-Universitat Central de Catalunya, 08500 Vic, Barcelona, Spain

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Correspondence to: Montserrat Serra-Millàs, PhD, Osona Mental Health, Hospital Universitari de Vic, Salut Mental i Innovació Social (SaMIS), Universitat de Vic-Universitat Central de Catalunya, C/Francesc Pla «El Vigatà» nº 1, 08500 Vic, Barcelona, Spain. serra2mont@yahoo.es
Telephone: +34-93-8831111
Fax: +34-93-7027786

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Abstract

Brain-derived neurotrophic factor (BDNF) plays an important role in central nervous system development, neurogenesis and neuronal plasticity. BDNF is also expressed in several non-neuronal tissues, and it could play an important role in other processes, such as cancer, angiogenesis, *etc*. Platelets are the major source of peripheral BDNF. However, platelets also contain high amounts of serotonin; they express specific surface receptors during activation, and a multitude of pro-inflammatory and immunomodulatory bioactive compounds are secreted from the granules. Until recently, there was insufficient knowledge regarding the relationship between BDNF and platelets. Recent studies showed that BDNF is present in two distinct pools in platelets, in α-granules and in the cytoplasm, and only the BDNF in the granules is secreted following stimulation, representing 30% of the total BDNF in platelets. BDNF has an important role in the pathophysiology of depression. Low levels of serum BDNF have been described in patients with major depressive disorder, and BDNF levels increased with chronic antidepressant treatment. Interestingly, there is an association between depression and platelet function. This review analyzed studies that evaluated the relationship between BDNF and platelet activation and the effect of treatments on both parameters. Only a few studies consider this possible confounding factor, and it could be very important in diseases such as depression, which show changes in both parameters.

Key words: Platelets; Brain derived neurotrophic factor; Depression; Antidepressants; Biomarkers

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Core tip: Brain-derived neurotrophic factor (BDNF) is expressed in neuronal and non- neuronal tissues and is stored peripherally in platelets. Platelet BDNF is present in α-granules and cytoplasm and only BDNF of granules is released by agonist stimulation. Little is known about mechanisms related to BDNF release in human platelets. Depressive disorders are associated with BDNF and platelet dysfunction. Low levels of serum BDNF have been described in major depression and they increased

with antidepressant treatment. Only a few studies have evaluated the relationship between platelet activation and peripheral BDNF values. This review suggests that platelet reactivity may partly explain the alterations in BDNF.

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INTRODUCTION

Major depressive disorder (MDD) is a common and invalidating mental illness, with a global point prevalence of 4.7%[1]. Depressive disorder is one of the leading causes of disability, and it has been suggested to become the disease with the second highest burden[2]. The lack of diagnostic and treatment markers in depression is one of the most important problems in clinical practice. Researchers are deeply involved in the identification of validated markers, particularly biomarkers that could be useful in clinical practice. Evidence suggests that brain-derived neurotrophic factor (BDNF) is relevant in the pathophysiology of depression and that it may be more useful as a biomarker for diagnostic and prognostic purposes than the other potential biomarkers[3]. BDNF is expressed in the nervous system and in non-neuronal tissues. The relationship between both is inconsistent, and will be analyzed in this work. Another factor that it has not been studied extensively is the storage and release of BDNF from platelets. Furthermore, depressed patients exhibit enhanced platelet reactivity and increased expression of activation markers, which may influence the characterization of BDNF in depression. The following review will focus on the relationship between platelet activation and BDNF, particularly in depression. This review will examine current literature regarding this topic until August 2015.

BDNF

BDNF, a member of the nerve growth factor family, is expressed in the central and peripheral nervous system and is widely distributed across subregions of the hippocampus and the frontal cortex, brain regions that are of crucial importance in the regulation of emotion, learning and memory[4-8] . BDNF-containing secretory vesicles are present in both the axon terminals and dendrites of neurons[9]. BDNF is also synthesized and released by astrocytes[10]. BDNF plays an important role in central nervous system development, neurogenesis, neuronal survival, migration, cell differentiation, growth of axons and dendrites and synapse formation[11-14], as well as in the synaptic process of hippocampal long-term potentiation (LTP) that play an important role in memory[4,15]. It also provides protection against learning and memory impairments under conditions of chronic stress[16].

The BDNF gene encodes a precursor protein (prepro-BDNF) that is cleaved into the smaller 35-kDa precursor, proBDNF, in the endoplasmic reticulum. ProBDNF need to be folded correctly, sorted and transported to the appropriate subcellular compartment. ProBDNF moves *via* the Golgi apparatus into the trans-Golgi network, where two kinds of secretory vesicles are generated: Those of the constitutive secretory pathway and those of the regulated pathway whose secretion is activity-dependent. ProBDNF can then be further cleaved into mature BDNF (mBDNF)[9]. In neurons, both proBDNF and mBDNF are preferentially sorted and packaged into vesicles in the activity-dependent secretory pathway. Activity-dependent secretion is believed to be an important feature because it may reflect the nature of the nervous system to respond to and form synaptic modulations based on experiences, and may be a cellular manifestation of memory and learning[17,18]. BDNF can also act *via* autocrine and paracrine mechanisms, depending on the site of the cell surface receptors through which it signals[19].

ProBDNF is either proteolytically cleaved by intracellular enzymes such as furin or pro-convertases and secreted as the 14 kDa mBDNF, or secreted as proBDNF and then cleaved by extracellular proteases[9]. The extent of the intracellular and extracellular processing of proBDNF is not exactly clear, but proBDNF is less efficiently processed by intracellular proteases compared to other neurotrophins, and the secretion of proBDNF seems to prevail over mBDNF[20-22]. It becomes important to identify the specific extracellular proteases that cleave proneurotrophins and understand their regulation. Several matrix metalloproteinases (MMP), including MMP3 and MMP7, have been shown to cleave pro nerve growth factor and proBDNF[23]. However, the most significant protease that cleaves proneurotrophins is the serine protease plasmin[23,24], which is generally expressed as an inactive plasminogen that must be activated by proteolytic cleavage by tissue plasminogen activator (tPA). In the brain, plasminogen is exclusively expressed in neurons and is present in the extracellular space, particularly at the synaptic cleft. tPA is secreted from axon terminals into the extracellular space, and this secretion depends on high-frequency neuronal activity[25]. Therefore, it is conceivable that tPA is the key trigger for the tPA-plasmin-proneurotrophin cascade. The regulation of MMP and plasmin expression or activation could regulate neurotrophin signaling in a spatially and temporally controlled manner. Other work has suggested that proBDNF (35 kDa) and tPA are secreted in an activity-dependent manner, and the extracellular conversion of proBDNF to mBDNF by the tPA/plasmin protease system is critical for late-phase LTP[24-26].

ProBDNF is not an inactive precursor and has been shown to have effects in the central nervous system

that are independent of mature BDNF, as it acts at a separate receptor. Once released, proBDNF preferentially binds to the pan neurotrophin receptor p75 (p75NTR), and mBDNF preferentially binds to both pre- and post-synaptic tropomyosin-related kinase receptors (TrkB), activating different intracellular secondary messenger cascades and affecting distinct cellular responses[27]. The binding of BDNF with TrkB results in intracellular phosphorylation and the activation of intracellular signaling cascades that trigger the so-called pro-survival pathways, inactivate pro-apoptotic signaling and promote neurogenesis[8,28]. ProBDNF binds to p75NTR, which leads to apoptosis and initiates long-term depression of synaptic transmission[29], causing a reduction in the complexity and density of dendritic spines in hippocampal neurons. Proteolytic cleavage of proBDNF represents an important mechanism by which the opposing cellular actions of proBDNF and mBDNF may be regulated[25].

PERIPHERAL BDNF

Platelets are the major source of peripheral BDNF[30,31], and they are important for storing the BDNF that is secreted from other tissues[32,33]. The BDNF and TrkB mRNAs are expressed in several non-neuronal tissues, including muscle, thymus, heart, liver, vascular smooth muscle cells, lung and spleen[34-38]. BDNF is also produced in monocytes, lymphocytes[39,40] and eosinophils. The latter cells produce BDNF *via* the autocrine system and utilize it to evoke and extend the allergic reaction[41,42]. BDNF has been shown to play a pivotal role in the growth, survival and chemoresistance of tumor cells in various types of cancers, including Hodgkin lymphoma, myeloma, hepatocellular carcinoma and neuroblastoma[43-47]. BDNF also mediates the survival and activation of endothelial cells through its interaction with TrkB[48-50], suggesting its potential role in angiogenesis. Many non-neuronal cells, such as smooth muscle cells, fibroblasts and astrocytes, may not express the molecular components of the regulated secretory pathway and therefore only secrete neurotrophins constitutively.

PLATELETS

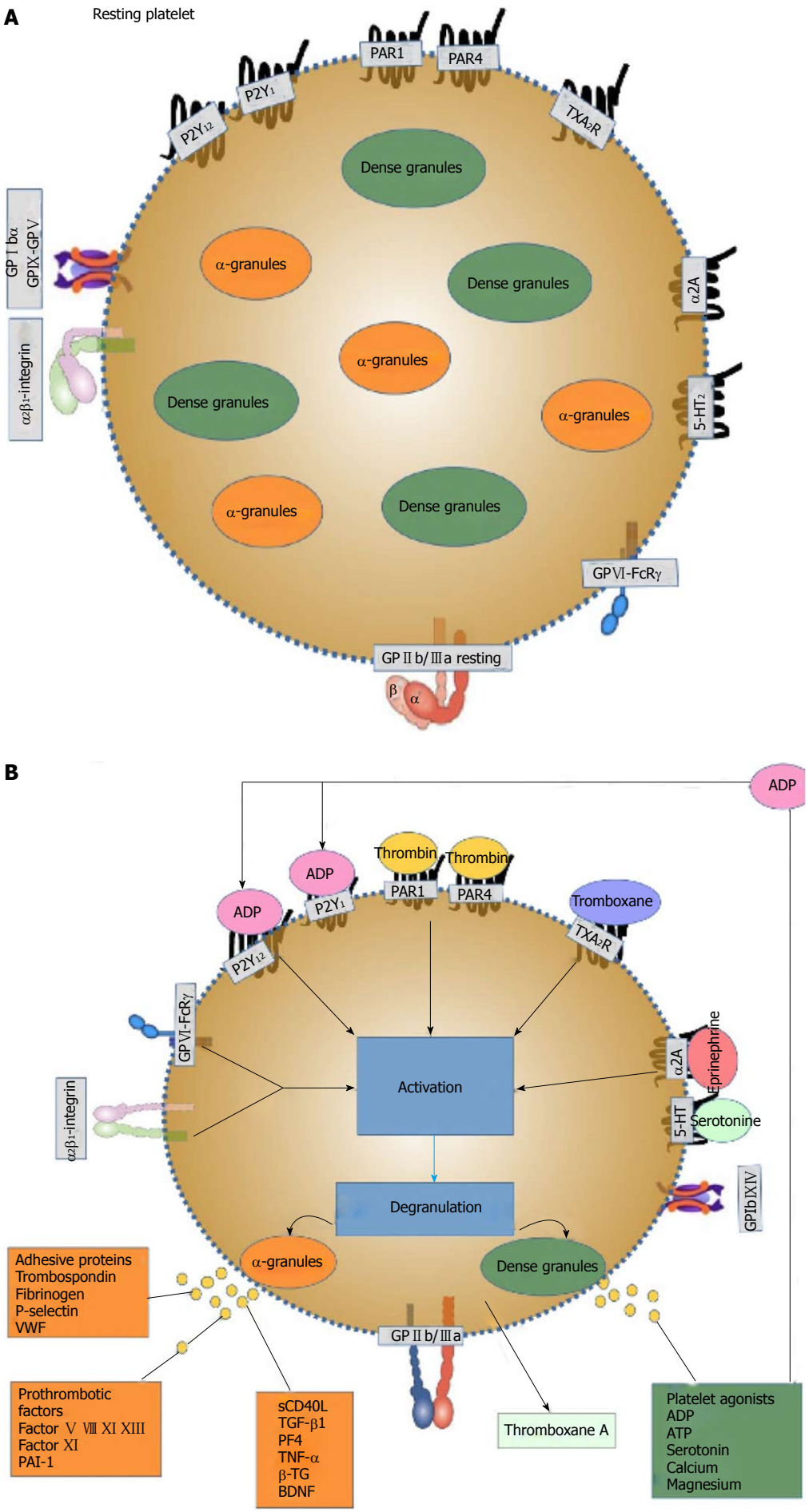
Platelets are small unnucleated blood cells with a size of approximately 3 μm that originate from megakaryocytes (MK) in the bone marrow, from which they are released into the blood system. They circulate for an average of seven to 10 d. Platelets contain many structures that are critical to stop bleeding. They contain proteins on their surface that allow them to adhere to breaks in the blood vessel wall and each other. They possess several important organelles: Three types of platelet secretory granules (α-granules - the most abundant, dense granules, and lysosomes), an open canalicular system and proteins similar to muscle proteins that allow them to change shape when they become sticky[51,52]. The platelets' organelle content is primarily taken up from the plasma; however, platelets are also able to synthesize molecules, such as platelet-derived growth factor (PDGF), platelet factor 4 (PF4), β-thromboglobulin (β-TG) and thrombospondin[53]. Figure 1A shows a resting platelet.

α-granules are essential for normal platelet activity. In platelets, the α-granules fuse with the plasma membrane upon activation, releasing their cargo and increasing the platelet surface area. N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) and SNARE accessory proteins control α-granule secretion, and hundreds of bioactive proteins are released from α-granules. The presence of distinct subpopulations of α-granule suggests that platelets may regulate the differential release of distinct classes of α-granules. This breadth of proteins implies a versatile functionality, and the α-granules participate in thrombosis and hemostasis, inflammation, atherosclerosis, antimicrobial host defense, wound healing, angiogenesis, and malignancy. Many aspects of the formation, structure, content, protein sorting, transport and release of α-granules are poorly understood[54]. Figure 1B and C show stimulated platelets.

Platelets are an essential component of the hemostatic process. Platelets must have additional roles in several physiological and pathophysiological regulatory processes[55] . They express many immunomodulatory molecules and cytokines, and they have the ability to modulate the immune system through interactions with various cells[56]. The binding of biochemical agonists (thrombin, collagen, ADP, epinephrine, arachidonic acid) or mechanical disruption of blood vessels initiates localized hemostatic responses that involve interactions of the vascular endothelium, platelets, red blood cells, coagulation and fibrinolysis. Platelet thrombus formation involves three main steps: Platelet adhesion to the damaged endothelium, activation, and aggregation. Platelet activation involves the generation of intracellular chemical signals that are initiated by platelets through specific surface receptors (platelets express certain surface markers, such as the active form of the glycoprotein receptor GPⅡb/Ⅲa, P-selectin and CD40 ligand). These signals cause dramatic morphological changes, such as the extension of pseudopodia, platelet-platelet aggregation, and granule secretion, resulting in the generation of a platelet thrombus.

BDNF IN PLATELETS

Ninety percent or more of blood BDNF is stored in platelets[32]. A close relationship has been found between BDNF and platelets under physiological conditions, where platelets are an important source of BDNF storage. In addition, there is an approximately 100- to 200-fold difference between the plasma and serum levels of BDNF because platelets release BDNF during the clotting process[32,57]. It has been elegantly demonstrated that the amount of BDNF in serum is nearly identical to the


A
Resting platelet
PAR1
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Dense granules
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Dense granules
GP Ⅰ bα
GPⅨ-GPⅤ
αIIbβ3-integrin
α2A
5-HT2
GPⅥ-FcRγ
GP Ⅱ b/Ⅲa resting
β
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PAR4
P2Y1
P2Y12
Tromboxane
TXA2R
GPⅥ-FcRγ
αIIbβ3-integrin
Activation
α2A
Epinephrine
5-HT
Serotonine
GPIbⅨⅤ
Degranulation
α-granules
Dense granules
GP Ⅱ b/Ⅲa
Adhesive proteins
Trombospondin
Fibrinogen
P-selectin
VWF
Prothrombotic factors
Factor Ⅴ Ⅷ Ⅺ ⅩⅢ
Factor Ⅺ
PAI-1
sCD40L
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β-TG
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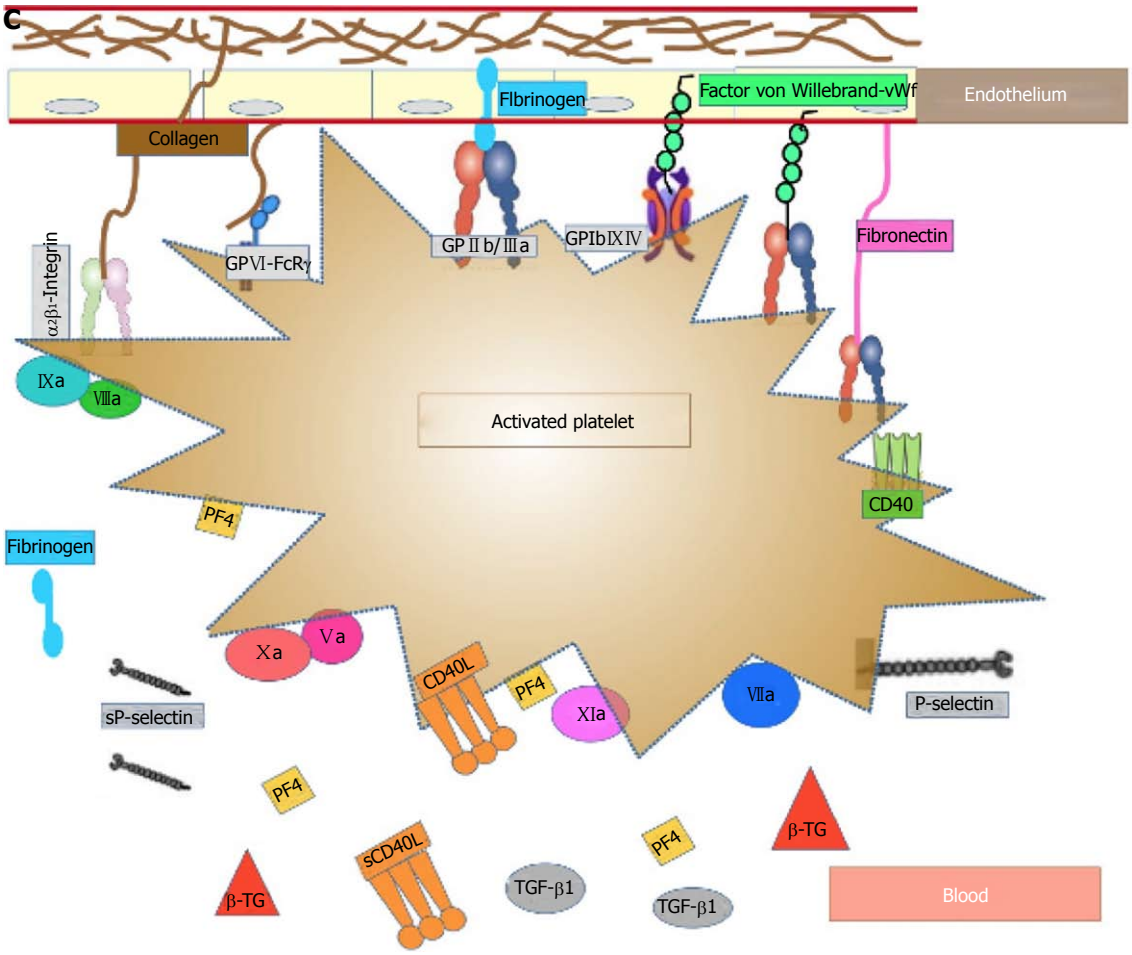

Figure 1 Representation of platelets before and after activation. A: Resting platelet: The discoid form of resting platelets is supported by a circumferential coil of microtubules lying just below the surface membrane whereas α-granules and dense bodies are irregularly dispersed in the cytoplasm. Platelet membrane contains receptors for ADP, thrombin, vWF, fibrin, fibronectin, epinephrine, PAF, thromboxane A2, Prostacyclin, trombospondin, serotonin and gycosil transferase; B: Platelet activation: Platelets may be activated *via* different agonists, through several receptor-mediator pathways (thrombin, TXA2, collagen, epinephrine, *etc.*). Collagen exposed within the denuded area of vascular endothelium stimulates platelet activation and adhesion to the vessel wall. These agonists bind and activate their respective receptors, which in turn stimulate the activation of associated G-proteins, ultimately activating GP IIb/IIIa receptors and promoting the interaction of adjacent platelets within the clot. When the platelet is stimulated a transient influx of calcium occurs, followed by extrusion of platelet storage granule contents. The dense bodies contain serotonin (5-HT), ADP, ATP and calcium, whereas the α-granules contain the highest amount of proteins, including vWF, fibronectin, fibrinogen, P-selectin, PF-4, β-TG, RANTES, CD40, CD40L, PDGF, amyloid precursor protein, MMP, adhesion molecules (ICAM, VCAM and PECAM), various coagulation and growth factors (such as BDNF) and inflammatory markers. All these phenomena are calcium (Ca^{2+})-dependent, amplify platelet activation and induce irreversible platelet-platelet aggregation and thrombus formation; C: Platelet activation-aggregation: Platelet aggregation following platelet activation is marked by a shape change that increases their surface area available for adhesion and gives platelets the ability to bind fibrinogen and vWF *via* the active form of the surface glycoprotein GP Ⅱb/Ⅲa receptors, leading to platelet aggregation and thrombus formation. Platelets express certain surface markers such as P-selectin and CD40 ligand and these expressed activation markers are cleaved, promoting the circulation of soluble CD40L and soluble P-selectin. Platelets also expose phosphatidylserine providing attachment sites for coagulation factors. Vascular injury also exposes subendothelial tissue factor, which forms a complex with factor Ⅶa and sets off a chain of events that culminates in the formation of the prothrombinase complex. Prothrombin is converted to thrombin, which subsequently converts fibrinogen to fibrin, generating a fibrin-rich clot. The coagulation cascade contributes to the stabilization of the thrombus. BDNF: Brain-derived neurotrophic factor; ADP: Adenosine-diphosphate; GP: Glycoprotein; ATP: Adenosine-triphosphate; PF-4: Platelet factor-4; β-TG: β-thromboglobulin; PDGF: Platelet-derived growth factor; vWF: Von Willebrand factor; MMP: Matrix metalloproteinases.

amount of BDNF found in washed platelet lysates[32]. Thus, the difference between the serum and plasma BDNF levels seems to reflect the amount of BDNF stored in circulating platelets, and the BDNF in platelets might serve as a reservoir for circulating BDNF. The BDNF in platelets may play a role during tissue trauma or nerve injury, releasing their contents into the circulation at the site of the injury[58].

In the first studies, BDNF was not expressed in or produced from the megakaryocyte precursor cell of the mature platelets, in which protein synthesis is generally absent, but was sequestered from the circulation[32,34]. Recently, one study found that BDNF is present in the cytoplasm of platelets and in α-granules, suggesting that BDNF is either produced in platelets or passed down from MK[59]. A second study found that a megakaryocyte progenitor line (MEG-01) produces BDNF upon thrombopoietin stimulation, also expressed in the bone marrow from the liver and kidney[60], and the levels of BDNF in MEG-01 cells increased in a time-dependent mannerly[61]. It was the first report of the production of BDNF in a megakaryocyte cell line and led to the

hypothesis that BDNF potentiates the cell proliferation of the megakaryocyte lineage *in vivo*. It is likely that there is a receptor for BDNF on the MEG-01 cell surface, but the TrkB receptor was not detected in MEG-01 cells or human platelets[32,59,62]. Therefore, there should be an unidentified novel receptor in MKs or platelets.

Some agonists, such as thrombin, collagen, Ca^{2+} and shear stress, could induce a rapid release of BDNF from platelets. Even with agonist stimulation, only approximately half of the BDNF in platelets is secreted, which suggests that platelets maintain a stable pool of BDNF as a buffer system[30]. However, another study found that platelet-mediated BDNF release in depressed patients was independent of platelet reactivity[31]. There was little knowledge about the relationship between BDNF and platelets and only a few studies have assessed issues such as platelet activation mechanism that allows the release of BDNF and BDNF localization within platelets.

The rate of BDNF release paralleled the secretion of 5-HT from the dense granules and PF4 from the α-granules, although a greater proportion (90%) of the total 5-HT and PF4 were released compared to BDNF. Because only 40%-60% of the total content of platelet BDNF was released by maximal platelet activation, some authors postulated that platelets either have a non-releasable pool of BDNF, or that the released BDNF is sequestered by binding to a transporter or receptor on the platelet surface[32]. Such binding could promote the internalization of BDNF by the platelets, as has been reported for 5-HT[63,64] and for astrocyte recycling of BDNF[65,66]. The binding of BDNF to washed platelets was confirmed by microscopy and FACS analysis, as well as confocal microscopy, suggesting that platelets bind exogenous BDNF[32]. However, a recent study found two different locations where it is stored BDNF: In the α-granules and in the cytoplasm. Using immunoelectron microscopy, BDNF was clearly detected in the same fractions as P-selectin, an α-granule marker, and protein kinase C, a cytoplasmic marker[59].

BDNF is predominantly released from the platelets through protease-activated receptor 1 (PAR1) activation during thrombin stimulation, along with vascular endothelial growth factor but not endostatin stimulation. Platelets stimulated with concentrations of the PAR1-activator peptide showed a dose-response curve of BDNF release, exhibiting a two-phase pattern. The first phase is a drastic release phase at low level activation which is completely inhibited by Prostaglandin (PGE1) pretreatment suggesting that this phase depends on calcium mobilization. The second phase is a mild release phase at high level activation which is not affected to PGE1 pretreatment, suggesting another signal that is not affected by PGE1[59]. BDNF response curve was similar to that of PF4[59]. There was no significant difference in BDNF release between the non-stimulated and PAR4-AP-stimulated cells. Interestingly, PAR1 activation promotes the release of proangiogenic factors and these results support the action of BDNF as a proangiogenic factor[47-50,67,68].

Another important finding of this study is that the BDNF in the α-granules is released upon platelet activation, whereas the cytoplasmic BDNF is not[59]. The maximum BDNF release is approximately 30%-40% with stimulation and the remaining 70% of BDNF is equivalent to that found in the cytoplasm, which is not released, similar to study of Fujimura[32].

MEASUREMENT OF BDNF IN PERIPHERAL SAMPLES

BDNF levels have been linked with various diseases, such as depression[69,70], schizophrenia[71,72], Alzheimer's disease[73] and anorexia[74]. Some available non-invasive options for evaluating BDNF include measuring the BDNF concentrations in the whole blood, serum, plasma and platelets. The results of BDNF in one sample cannot be directly generalized to other peripheral BDNF parameters because there are only modest statistically significant associations among these peripheral measurements. Studies found that the correlations between the plasma and serum BDNF concentration are between r = 0.2 to r = 0.70[75-77]. The distinct biological significance of the serum and plasma BDNF levels has already been noted[78,79].

Human serum contains BDNF at far higher concentrations than human plasma. Because of the storage of BDNF in platelets, the concentrations of BDNF in serum and plasma differ by a factor of 200[58]. There are some confounding factors regarding the serum BDNF levels. Serum BDNF showed strong associations with race, platelet count, and depression after adjusting for other factors. Females, blacks, smokers, and those with high platelet counts had higher serum BDNF levels[80]. The platelet count is considered the factor with the strongest association with the serum BDNF concentrations[81,82] and this strong association is consistent with theory that the BDNF in serum is derived directly from the platelets[80]. The serum BDNF concentrations largely reflect the activation-dependent release of BDNF from platelets[32,57]. Interindividual differences in the serum BDNF concentrations are mediated by changes in the activity of the blood platelets caused by medications or pathological conditions[83].

The BDNF protein circulates in plasma for less than an hour[84,85]. The measures of BDNF in plasma samples are quite unstable, and previous studies reported a large degree of dispersion, which could reflect the different cellular sources of plasma BDNF (endothelia, central nervous system, *etc.*) and the influence of different factors on the expression and secretion of BDNF from different sources[57]. Lower levels in males was observed and age, body weight and cholesterol-LDL showed a negative correlation with the plasma BDNF levels[57]. This study showed that the BDNF plasma levels significantly decreased with increased age or weight, whereas the platelet counts did not. However, one study

found that the platelet count is the most important predictor of plasma BDNF concentrations in children and adolescents, and the plasma BDNF concentrations in children may need to be interpreted with age-specific and platelet count-specific standards[86]. Another study found the plasma BDNF levels were positively associated with the platelet count and negatively associated with the fibrinogen level in patients with angina pectoris. Similar to BDNF, fibrinogen is a major storage protein of the platelet α-granules and is delivered to the α-granules by endocytosis. These associations may be associated with the level of BDNF release from platelets in inflammatory states, and the low plasma BDNF concentration in patients with angina pectoris may be primarily due to platelet release[87].

Another consideration is that the plasma BDNF levels have a very low retest stability when measured twice within one year, and there are substantial changes, even throughout one day. Recently, one study raised methodological concerns regarding the assessment of the BDNF levels in plasma and recommends measuring the BDNF levels in serum[88]. However, the plasma BDNF levels could represent the currently biologically active BDNF as a state-dependent marker, and it appeared to be influenced by inflammation mediators[89].

Platelets circulate for up to 11 d in the peripheral blood[84,85]. The platelet BDNF levels could represent a long-term marker of the varying plasma BDNF levels over a period of several days. The BDNF stored in platelets is likely obtained from both the circulating plasma pool, from cells in the brain or other organs and from megakaryocites. Variation in BDNF production in specific organs[57] may produce changes in platelet BDNF content and it measure could be a circulating indicator of altered production. Differences in platelet function and release of or sequestering BDNF from the blood may result in the differences between the serum and plasma BDNF concentrations[70]. The platelet and serum levels have been shown to strongly correlate, and it has been proposed that the BDNF released from platelets directly correlates to the serum BDNF levels; however, in recent studies, only 30% of the BDNF in platelets is secreted into the blood[59]. The platelet BDNF levels did not correlate with age, weight, cholesterol or long-term storage but changed with menstrual cycle[57,90].

Another limitation of measuring BDNF in the periphery is that the ELISA kits used in most studies quantified the total BDNF concentrations in serum and did not make the distinction between the pro- and the mBDNF variant, but the two BDNF variants are functionally different. Moreover, this raises the question of which parameter best serves as a mirror for the neurotrophic action in the brain. Some authors have suggested that the leukocyte BDNF mRNA content could more closely reflect central BDNF dynamics because of its short half-life[91] and therefore may be less subject to the peripheral confounding factors. In addition, it has been argued that a combination of peripheral BDNF indices may have advantages over a single index. Assessing both the platelet and serum BDNF concentrations could be particularly relevant[3].

CORRELATIONS BETWEEN THE CEREBRAL AND PERIPHERAL BDNF LEVELS

Because the central BDNF levels are difficult to obtain for methodological and ethical reasons, there is a great interest in peripheral BDNF measures in relation to psychiatric illness. There are indications that the BDNF measured in peripheral tissues reflects BDNF activity in the brain. These indications include preclinical findings that BDNF crosses the blood-brain barrier[92] and positive correlations between the peripheral and central BDNF concentrations[57,93,94]. Referring to the blood-brain barrier, some rodent studies have shown that peripheral BDNF administration promotes the regeneration of spinal cord injury[95], has an effect on depressive-like behaviour[96] and could increase BDNF levels in the brain[97]. Other studies did not find these results but found that BDNF protein may have poor or null blood-brain barrier penetrability[98-100]. Systemically administered BDNF in rodents showed that BDNF signaling pathways were activated only in disrupted regions[101]. However, studies also point towards a role for vascular endothelial impairment in MDD[102]. A meta-analysis found an increased risk of MDD in those with major vascular diseases including diabetes, cardiovascular disease and stroke[103].

Evidences regarding the positive correlation between the peripheral and central BDNF concentrations[57,93,94] are as follows. Human post-mortem studies had indicated similar alterations in BDNF concentrations in the brain and periphery of persons who were depressed at the time of death[104]. In one human study, the BDNF levels were higher in blood that was derived from the internal jugular veins compared to the arterial blood[105], suggesting that the source of BDNF in the peripheral tissues can be found in the brain. These studies indicated that neurotrophic function can be estimated from the periphery in a rather non-invasive manner by taking advantage of this "window to the brain". However, the specificity, extent and relationship between the peripheral BDNF levels to disease activity are not fully known. Due to the large variation in the amplitude between the serum and plasma BDNF levels, it is unlikely that it will translate as a useful biomarker of disease activity in clinical practice[106]. Future studies should determine the ratios of the BDNF concentrations in the cerebral spinal fluid (CSF), serum and plasma in acutely ill and remitted patients and controls.

Other studies report null findings with regard to an association between the peripheral BDNF concentrations and the more central parameters for BDNF activity, namely an absence of correlations between the plasma and CSF concentrations of BDNF[107]. In an older

adult study, blood-based measures of BDNF are not representative of the CSF BDNF levels[80]. A study comparing the CSF and serum BDNF measurements in a sample of patients with Alzheimer's disease found that they were not correlated[73]. Moreover, the concentrations of BDNF are much higher in the serum (> 1000 ×) or plasma (approximately 10 ×) than in cerebrospinal fluid, which may reflect peripheral synthesis[106-108]. One explanation is that tissues other than the brain, including the immune system, liver, smooth muscle and vascular endothelial cells serve as sources of BDNF[34,35]. Neurons and glia cells of the central nervous system might originate a substantial portion of the circulating BDNF, but our current knowledge does not allow us to distinguish whether the peripheral sources produce or release less BDNF or if decreased synthesis or release in the brain are responsible for the lower plasma and serum levels[92,106]. Another explanation is that these changes in the BDNF levels may represent a counter-regulatory response to other etiological factors of the illness, such as metabolic and redox factors[109]. Another reason to criticize this relationship between the brain and peripheral BDNF levels could be that the expression of BDNF is specific to a particular local and time[110], and animal studies have shown that the levels of BDNF are increased in some brain regions and decreased in others in some diseases[111].

RELATIONSHIP BETWEEN BDNF AND PLATELET ACTIVATION

The relationship between platelet activation and the peripheral BDNF level is poorly documented in humans. No evidence shows the interaction between BDNF and platelets under pathological conditions, such as tumor growth and metastasis[32]. Therefore, we will review the studies examining the relationship between BDNF and different markers of platelet activation.

Transforming grown factor β1

Transforming grown factor β1 (TGF-β1) is abundant in platelets and is stored in the α-granules[112]. It plays an important role in regulating the immune response, cell proliferation and tissue fibrosis, and it recruits inflammatory cells to the wound area. Lommatzch *et al*[57] found a stronger correlation between the serum BDNF levels and the serum TGF-β1 levels than with the serum 5-HT levels and the first two could be anatomically and functionally related in the platelet both located in α-granule. The postoperative abdominal surgery changes in the serum BDNF levels virtually paralleled the changes in platelet number and the platelet mediator TGF-β1. The platelet numbers and TGF-β1 concentrations decreased in the immediate acute response and increased later. A positive relationship was observed between the serum BDNF and TGF-β1 values at all times after surgery[89].

Fibrinogen

Similarly to BDNF, fibrinogen is a major storage protein of platelet α-granules and is delivered to the α-granules by endocytosis[113]. Recent studies reported an association between elevated plasma fibrinogen levels and psychological distress and depression in individuals from the general population after adjusting for confounders[114], but the effect size of plasma fibrinogen could be small[115]. Another study found higher fibrinogen levels in non-responders than in responders in major depression patients, suggesting that baseline plasma fibrinogen levels can serve as a biomarker to gauge the success of antidepressant treatment response[116]. Hattori *et al*[115] found that a subpopulation of patients with MDD had high CSF fibrinogen levels compared with controls and those patients with a high fibrinogen level had white matter tract abnormalities. The increased CSF fibrinogen in patients could represent a trace of blood-brain barrier disruption induced by neuroinflammation, which is in accordance with the mild inflammation hypothesis in the aetiology of MDD[117,118]. The plasma BDNF levels were negatively associated with the fibrinogen levels in patients with angina pectoris[87]. The exact reason for the association between the decreased plasma BDNF levels and these factors is unclear, but it may be related to the level of BDNF release from platelets in inflammatory states.

P-selectin

P-selectin (CD 62-p) is primarily located in the α-granule membrane of resting platelets and is only found on the platelet surface after platelet activation. P-selectin is perhaps the most cited of the biologically active molecule that appears on the platelet surface after activation and secretion. P-selectin on platelets or endothelial cells has a key role in inflammation. The soluble P-selectin (sP-selectin) levels in blood represent a measure of platelet and/or endothelial cell activation. Elevated sP-selectin levels were associated with enhanced generation of tissue factor-expressing microparticles, leading to shorter plasma clotting time and a pro-coagulant phenotype, which facilitated fibrin generation and also altered the blood-brain barrier permeability and exacerbated stroke[112]. One study in cardiac patients showed that the median serum BDNF levels were higher in the myocardial infarction (MI) group than in the stable angina pectoris (SAP) group. In the MI patients, there was a significant correlation between the BDNF and sP-selectin levels. In contrast, no such correlation was observed in the SAP patients. The study suggested that the BDNF serum levels in MI patients could be related to platelet activation and the inflammatory response[119].

Soluble CD-40-ligand

CD40L appears to be localized to the granule membranes. Upon platelet activation, it is translocated to the platelet surface, where it is cleaved and acts by associating with the αⅡbβ3 integrin. This soluble form

[soluble CD-40-ligand (sCD40L)] is predominantly derived from activated platelets and thus represents a circulating marker of platelet activation. Platelets are the main source of sCD40L and are responsible for > 95% of the circulating sCD40L levels. A study by Lorgis *et al*[119] in patients with coronary artery disease did not find a significant correlation between the serum BDNF levels and sCD40L in either the MI or SAP patients. The controversial results between the sCD40L and sP-selectin levels could be explained by methodological issues regarding the measurement of both markers[120,121].

PF4

PF4 is a platelet-specific protein that is stored in the α-granules and secreted into the plasma upon platelet activation[64]. PF4 is considered an index of platelet reactivity. Patients with depression showed increased PF4 plasma levels with respect to the controls, but there were no differences in the serum PF4 levels. The total PF4 levels obtained by completely clotting the platelets are the same in both groups. Alterations in the serum and plasma BDNF levels in depression are not related to the changes in either the whole blood BDNF levels or in the platelet release of the activation marker, PF4[31]. The plasma PF4 levels were elevated, indicating increased platelet reactivity without a change in the total PF4 levels in the serum. These results suggested that there are independent regulatory mechanisms for platelet BDNF and PF4 release. However, one question is whether it is possible for two proteins that are supposed to be stored in the same granules to be independently released or if there are different subcellular localizations for these proteins. Another study found that the rate of BDNF release paralleled the secretion of 5-HT from the dense granules and of PF4 from the α-granules, although a greater proportion (90%) of the total 5-HT and PF4 were released compared to BDNF[32].

β-TG

The plasma β-TG levels were significantly decreased in patients with Alzheimer's disease compared to the healthy controls. In Alzheimer's disease patients, the serum BDNF concentrations were significantly correlated to the β-TG and plasma BDNF values. In contrast, the plasma BDNF and β-TG values were not significantly correlated. The levels of BDNF and β-TG in the blood of patients with Alzheimer's disease are decreased compared to the controls. These results confirm an association between the serum BDNF concentration and the degree of platelet activation, as measured by the plasma β-TG levels[122].

EFFECTS OF ANTIDEPRESSANTS OR ANTI-PLATELET DRUGS ON BDNF RELEASE FROM PLATELETS

The release of BDNF from platelets is a polemical issue. It seems likely that methodological differences may markedly affect the results, including the methodology for isolating the platelets (pH, use of diverse inhibitors, *etc.*); anticoagulation (using EDTA/citrate tubes, heparin a, *etc.*); buffers with or without calcium; acute/chronic treatment; receptor profile or mechanism of action of the drug; drug doses; animal or human studies; and direct study of platelet BDNF levels or calculating the difference between the serum and plasma BDNF levels. For example, an elevation in the calcium concentrations plays an important role in platelet activation and secretion[123]. High concentrations of calcium and thrombin lead to an immediate release of diverse growth factors, including PDGF and TGF-β1[124,125]. Under calcium-free conditions, only a low amount of 5-HT and BDNF (approximately 10%-16% of the total content) was released after 10 and 60 min; however, almost all of the nerve growth factor was released[126,127].

Animal studies and single doses of an antidepressant

One study investigated the direct influence of antidepressants on BDNF release from platelets and their effects on the serum levels. Platelet BDNF release was studied using samples of washed platelets prepared from rat blood that they had incubated with sertraline, paroxetine, fluvoxamine and milnacipran, and the BDNF levels were determined at different time points. The changes in the serum BDNF concentrations were studied after single intravenous injection of antidepressants in rats at 1, 2 and 5 h following the injection. The BDNF from platelets was released by these antidepressants, and there were no differences between the effects of serotonin-norepinephrine reuptake inhibitors and selective serotonin re-uptake inhibitors (SSRIs). Antidepressants promoted BDNF release from platelets within 1 h, and the changes in BDNF release depended on the amount of the antidepressant and they were specific for each antidepressant. Sertraline was the most effective antidepressant in promoting platelet BDNF release[128], but other study with human controls did not found it[129]. The BDNF released represented approximately 20% of the total BDNF in platelets, similar to other agonists[32,59]. The serum BDNF concentration increased 1 h after sertraline injection; this change exhibited a significant difference at 5 h and was dose-dependent from 0.03 μmol/L to 0.3 μmol/L of sertraline in rat platelets. BDNF release from platelets is affected by antidepressants, which means that the administration of antidepressants might affect the changes in the BDNF in the peripheral tissues, such as platelets, and the serum BDNF concentrations. The authors suggested that the decreased serum BDNF concentrations in depressed patients may reflect reduced platelet BDNF levels[128].

Another study showed that treatment of rat platelets with sertraline, citalopram, paroxetine and indomethacin did not influence the release of BDNF after 10 and 60 min independently calcium conditions. BDNF release was significantly reduced by ibuprofen, an anti-inflammatory drug, after 10 and 60 min[127] but only when calcium was present, similarly with the work of

Fujimura *et al*[32].

Glutamatergic modulator riluzole have been proposed as a strategy for the treatment of mood disorders. Riluzole could stimulate BDNF release, acting directly on these cells. When platelets of healthy controls were incubated with riluzole at low concentrations for 4 h, but not for 24 h, riluzole stimulated the release of BDNF. This acute effect, but not later, suggests that its effect derived from evoking neurotrophin release. Moreover, mean platelet volume and platelet distribution width did not change during study, so platelets used in this study were not activated at the time of exposure to riluzole[129].

Chronic antidepressant use in humans

One study evaluated the changes in the platelet BDNF levels in patients with major depression when they were treated with s-citalopram. The platelet BDNF levels of the untreated patients appeared significantly lower than those of the healthy subjects, and antidepressant treatment with an SSRI normalized the platelet BDNF levels. The platelet BDNF levels were normalized earlier (at eight weeks of treatment) than the plasma BDNF levels[70]. Another study evaluated the platelet BDNF levels in patients with MDD and childhood trauma. They were treated with antidepressant medications for three months, including escitalopram, mirtazapine, and duloxetine, without intensive psychotherapy. The platelet and serum BDNF levels showed a significant increase from baseline at the 3-mo follow-up in the patient group. Conversely, the plasma BDNF levels were not significantly different between the two time points in the patient group. There were no significant differences in BDNF levels between the different antidepressant treatment groups[76] in either of the peripheral samples analysed.

Anti-platelet regimens in humans

A study by Stoll *et al*[130] investigated the impact of common anti-platelet drugs on the BDNF concentrations in serum and plasma and on the release of BDNF from platelets in a group of healthy volunteers. They showed that a single oral dose of clopidogrel but not aspirin significantly reduced the release of BDNF from platelets in healthy volunteers. The platelet α-granule marker TGF-β1 was also significantly reduced in the serum after clopidogrel treatment but not after aspirin administration. In addition, the decrease in the serum TGF-β1 concentrations correlated with the decrease in the serum BDNF concentrations at 24 h after clopidogrel administration. Aspirin and clopidogrel had no significant effects on the plasma BDNF levels[130]. Another study found a reduction in the release of BDNF from platelets when treated with aspirin (a non-specific cyclooxygenase-inhibitor)[131]. These drugs act through different mechanisms; aspirin acts by inhibiting cyclooxygenases, and clopidogrel irreversibly binds to the membrane adenosine diphosphate receptor and impacts platelet α-granule degranulation[130]. The study by Stoll *et al*[130] is consistent with the decrease of the platelet α-granule marker TGF-β1 after clopidogrel treatment and the correlation between the effects of clopidogrel on the BDNF and TGF-β1 concentrations but not those after aspirin administration.

SIGNIFICANCE OF PLATELET BDNF RELEASE IN DEPRESSION

Many attempts have been made to generate reliable blood-derived candidate biomarkers based on the current models of disease pathogenesis. 5-HT and BDNF are known to modulate behavioral responses to stress and to mediate the therapeutic efficacy of antidepressant agents, and they interact at different levels[132]. BDNF has been implicated in the pathophysiology of depression[133], and it has been studied as biomarker of this disease. A large number of clinical studies have reported that the BDNF levels in serum[83,134-136] are significantly decreased in depressed patients and that this decrease is normalized by antidepressant treatments[135,137-140], which was confirmed by meta-analysis[141,142]. In some of these studies, the BDNF levels correlated with higher scores on scales for assessing depression[141], although there are studies that have found no such correlation[108,143]. A recent published meta-analysis[3] concluded that there are low concentrations of BDNF in the serum of patients with untreated depression, but the size of the effect becomes substantially smaller than in previous studies. However, no consistent associations were found between the serum concentrations of BDNF and the severity of the depressive symptoms. An aspect to consider is that the serum BDNF levels are dependent on the release of BDNF from platelets[32,57], which has not been evaluated in most studies. Another aspect to consider is that other diseases, such as schizophrenia, bipolar disorder, and anorexia, among others, have shown decreases in the serum BDNF levels; thus, this finding is not specific enough for a diagnostic marker.

With respect to the plasma levels, some studies showed lower plasma BDNF levels in depressive patients[78,144,145] or did not observe changes[146,147]. Our group reported that the plasma BDNF levels were significantly higher in the depressed patients compared to the healthy controls and that they were similar in both groups when the symptoms remitted[70]. One possible explanation could be that BDNF is released from platelets and increases the plasma BDNF levels. However, the BDNF in plasma has a short half-life, and platelets contain greater concentrations of BDNF than the plasma. The plasma BDNF levels also appeared to be influenced by inflammation mediators[89].

There are few studies that have evaluated the platelet BDNF levels in depression. Two studies that directly evaluated the platelet levels showed reductions in the platelet BDNF levels in depression, which may be associated with lower serum BDNF levels in patients

with major depression, but these two studies did not evaluate the effects of treatment[148,149]. Another study showed that the platelet BDNF levels were significantly decreased with respect to the controls, but treatment with SSRIs normalized the levels to the levels of the controls[70]. These data are consistent with a preactivation state of platelets in major depression. Treatment with an SSRI would improve this preactivated state and explain the increase in the BDNF levels inside the platelets[70]. The remaining studies evaluated the platelet BDNF levels indirectly by the difference between the serum and plasma levels[76].

A disruption in serotonergic signaling in the brain is believed to be involved in the pathophysiology of depression. It is well known that 99% of the 5-HT found in the human body is stored in platelets and that 5-HT can induce downstream platelet aggregation and coronary vasoconstriction[150]. In contrast with arachidonic acid, 5-HT is a weak platelet agonist that requires co-stimulation with other agonists to induce full platelet activation[151]. Increasing evidence indicates that there is an association between depression and platelet function[152-154]. A relationship between depressive symptoms and increased platelet activity has been established in physically healthy depressed patients[155] and, in post-MI depressed patients[156]. Elevated platelet reactivity has been found in depressed patients, as indicated by the increased plasma levels of either PF4 or β-TG or the increased expression of procoagulant platelet surface receptors[157-159]. Our group demonstrated the existence of a prothrombotic endophenotype in the platelets of depressed patients before treatment that was characterized by a statistically significant increase in the average volume of platelets and high expression of glycoprotein GPIb and antigen markers. Compared to the controls, the patients' platelets showed a significantly enhanced aggregation response to arachidonic acid. The clot firmness and procoagulant activity of platelet-associated tissue factor were also significantly elevated, which can contribute to the increased prothrombotic profile of platelets and precipitation events in ischemic vascular lesion sites[160]. Studies with circulating blood revealed increased fibrin formation and thrombin generation in depression compared to the blood of healthy donors, when exposed to a thrombogenic surface in flow conditions[161]. Other observed alterations in platelet parameters in patients with major depression included a reduction of 5-HT transporter [3H]-imipramine binding sites in platelets[162] and increased in 5-HT_2 receptor binding sites on the platelet surface compared to the controls[163]. Platelet monoamine oxidase activity has been shown to be elevated in depressed patients[164]. Heightened membrane expression of glycoprotein Ⅱb/Ⅲa and the P-selectin receptors has also been reported in depressed patients without heart disease[156]. These alterations have been proposed as a possible mechanism that contributes to the elevated cardiac risk associated with the diagnosis of major depression[165]. Several mechanisms could explain the platelet abnormalities observed in major depression[159]. Although many studies have shown exaggerated platelet activation in patients with depression, several have shown no such relationship[152].

During platelet activation, 5-HT stimulation also accelerates the exocytosis of the platelet α-granules, which secrete procoagulant molecules into the plasma. One of these molecules is plasminogen activator inhibitor-1 (PAI-1), which is released at the site of thrombus formation. The levels of PAI-1 in arterial clots are 2-3 times higher than those observed in venous clots, and the relative content of PAI-1 determines the resistance to thrombolysis. PAI-1 inhibits the bioavailability of tPA and plasmin, which are the proteases that cleave proBDNF to mBDNF; therefore, the elevated synthesis of PAI-1 reduces the production of mBDNF. Multiple lines of evidence have shown that split proBDNF is central to the pathophysiology of major depression and the mechanisms of action of antidepressants[166]. The inadequate split of proBDNF may increase the risk of mood disorders[167]. In fact, patients with major depression show increased levels of proBDNF and decreased levels of mBDNF[168]. Moreover, PAI-1 inhibits the production of plasmin, preventing the dissolution of blood clots in atherosclerotic plaques, and one would expect that patients with depressive disorders have a higher risk of cardiovascular events.

In platelets, SSRIs results in a decrease in the 5-HT storage in dense granules, and thus could affect platelet aggregation[169]. 5-HT can definitely potentiate platelet mediated thrombogenesis[151]. Continued treatment with the SSRI may modulate not only the circulating levels of 5-HT but also the presence and activity of the serotonergic mechanisms and inhibit the release of 5-HT during platelet aggregation[170,171]. Clinical data have also shown that antidepressant treatment influenced platelet activation by lowering the plasma PF4 and β-TG levels[158,159], but other studies did not find this association[172]. Citalopram inhibited human platelet aggregation *in vitro* in response to different agonists, with the strongest effect observed in response to ADP and ADP + 5-HT[151]. *Ex vivo* escitalopram treatment of blood resulted in a significant inhibition of ADP- or collagen-induced platelet aggregation[165,173,174]. In aggregation experiments with collagen or arachidonic acid, a significant decrease in the aggregation intensity was noted in the platelets of SSRI-treated patients[175]. The addition of ADP to platelets from patients treated with SSRIs induced an inhibition of ATP release and the secondary wave of platelet aggregation[176]. Furthermore, a significant decrease in the platelet activation markers CD62-P and annexin-V-binding was reported after preincubating platelets with citalopram, but the release of factor V/Va from the α-granules was not noticeably affected[151]. Treatment with SSRIs for 24 wk normalized the majority of the altered parameters in patients with depression, but it accentuated the

expression of GPⅡb/Ⅲa and the viscoelastic properties of the clots formed under low shear rate conditions[161]. SSRI treatment rapidly and effectively counteracted the enhanced fibrin or aggregate formation observed under flow conditions, confirming the previous *in vitro* results in the clinical setting[151]. However, the viscoelastic parameters showed a progressive acceleration of clotting time and enhanced clot strength during the treatment with escitalopram, but these results were obtained in almost static conditions[161]. Patients treated with SSRIs seem to have fibrinogen and PAI-1 levels in plasma that are similar to those of the healthy controls and lower than depressed patients who do not receive serotonergic antidepressants[177]. Overall, these results could explain why the patients treated with SSRIs show a reduction in cardiovascular disease risk when compared with patients not receiving antidepressants[178,179].

CONCLUSION

To the best of our knowledge, only a few studies have assessed both BDNF and platelet activation in depression[31]. BDNF has been implicated in the pathophysiology of depression[133], and it has been studied as a biomarker of this disease. A large number of clinical studies have reported that the BDNF levels in serum[83,134-136] are significantly decreased in depressed patients and that this decrease is normalized by antidepressant treatments[135,137-140], which was confirmed by meta-analysis[141,142]. Serum levels are influenced by platelets and plasma levels results are inconsistent. Ninety percent or more of blood BDNF is stored in platelets, but these studies did not consider the platelet alterations observed in depression. Increasing evidence indicates that there is an association between depression and platelet function[152-154], with an elevated platelet reactivity, a prothrombotic endophenotype and increased of plasma substance levels excreted from α-granules in depressed patients. Some authors propose that the lower peripheral BDNF concentrations in depression and their upregulation over the course of antidepressant treatment may be an epiphenomenon resulting from an altered BDNF metabolism or expression by these peripheral organs[3]. One possible explanation could be that alterations in peripheral BDNF levels in depression depend more on platelet reactivity that excretes more BDNF from α-granules than on alterations of central BDNF. A serious question remains to be answered of whether the relationships found between BDNF and depression may be mediated primarily by the relationship between depression and platelet activation. Further studies are required to evaluate the complexity of the relationship between BDNF and platelet reactivity and its possible influence on the peripheral levels in certain diseases, such as depression. Another group of studies is required to evaluate the implications of antiplatelet and antidepressant drugs in the relationship between BDNF and platelet activity.

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REVIEW

Biomarkers in schizophrenia: A focus on blood based diagnostics and theranostics

Chi-Yu Lai, Elizabeth Scarr, Madhara Udawela, Ian Everall, Wei J Chen, Brian Dean

Chi-Yu Lai, Elizabeth Scarr, Madhara Udawela, Ian Everall, Brian Dean, the Florey Institute of Neuroscience and Mental Health, Parkville, VIC 3010, Australia

Chi-Yu Lai, Wei J Chen, Institute of Epidemiology and Preventive Medicine, College of Public Health, National Taiwan University, Taipei 100, Taiwan

Chi-Yu Lai, Wei J Chen, Center of Genomic Medicine, National Taiwan University, Taipei 100, Taiwan

Elizabeth Scarr, Ian Everall, Brian Dean, Department of Psychiatry, the University of Melbourne, Parkville, VIC 3010, Australia

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Correspondence to: Chi-Yu Lai, PhD, Institute of Epidemiology and Preventive Medicine, College of Public Health, National Taiwan University, 17 Xu-Zhou Road, Taipei 100, Taiwan. jchiyulai@gmail.com
Telephone: +886-2-33668010
Fax: +886-2-33668004

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Abstract

Identifying biomarkers that can be used as diagnostics or predictors of treatment response (theranostics) in people with schizophrenia (Sz) will be an important step towards being able to provide personalized treatment. Findings from the studies in brain tissue have not yet been translated into biomarkers that are practical in clinical use because brain biopsies are not acceptable and neuroimaging techniques are expensive and the results are inconclusive. Thus, in recent years, there has been search for blood-based biomarkers for Sz as a valid alternative. Although there are some encouraging preliminary data to support the notion of peripheral biomarkers for Sz, it must be acknowledged that Sz is a complex and heterogeneous disorder which needs to be further dissected into subtype using biological based and clinical markers. The scope of this review is to critically examine published blood-based biomarker of Sz, focusing on possible uses for diagnosis, treatment response, or their relationship with schizophrenia-associated phenotype. We sorted the studies into six categories which include: (1) brain-derived neurotrophic factor; (2) inflammation and immune function; (3) neurochemistry; (4) oxidative stress response and metabolism; (5) epigenetics and microRNA; and (6) transcriptome and proteome studies. This review also summarized the molecules which have been conclusively reported as potential blood-based biomarkers for Sz in different blood cell types. Finally, we further discusses the pitfall of current blood-based studies and suggest that a prediction model-based, Sz specific, blood

oriented study design as well as standardize blood collection conditions would be useful for Sz biomarker development.

Key words: Schizophrenia; Peripheral blood; Biomarker; Diagnostics; Schizophrenia-associated phenotype; Treatment response; Clinical courses

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Core tip: In recent years, there has been search for blood-based biomarkers for schizophrenia (Sz) as a valid alternative. However, it must be acknowledged that Sz is a complex and heterogeneous disorder which needs to be further dissected into subtype using biomarkers. The scope of this review is to critically examine published blood-based biomarker of Sz, focusing on possible uses for diagnosis, treatment response, and their relationship with schizophrenia-associated phenotype. We suggest that a prediction model-based, Sz specific, blood oriented study design as well as standard blood collection procedures would be useful for development of Sz biomarkers.

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INTRODUCTION

Identifying biomarkers that can be used as diagnostics or predictors of treatment response (theranostics) in people with schizophrenia (Sz) will be an important step towards being able to provide personalized treatment and would support efforts to develop new drug treatments[1-3]. Sz is a psychiatric disorder and there have been great efforts to the study of potential neuronal and glial abnormalities that may provide the basis of the aetiology of the disorder. However, findings from such studies have not yet been translated into biomarkers that are practical in clinical use[4] because brain biopsies are not acceptable and neuroimaging techniques are expensive[5] and the results are inconclusive. Thus, in recent years, there has been search for blood-based biomarkers for Sz as a valid alternative[5,6].

Blood-based biomarkers are regarded as a feasible option because the dysregulation of gene expression, epigenetic patterns, protein quantities, metabolic and inflammatory molecules in peripheral blood have been shown to have distinct patterns in people with Sz[4,5,7-11]. In addition, the strong heritability of Sz suggests that there may be genetic markers detectable in peripheral tissue[12]. Finally, there are data that suggest changes in gene expression[13,14], epigenetic patterns[15], proteomic/metabolic markers[16-18] and functional cellular pathways[16,19-21] are present in both the peripheral and central nervous system (CNS) tissue. More recently our concepts about the interactions between the brain and periphery have been expanded with data suggesting that the CNS may influence gene expression and metabolism in the peripheral blood *via* cytokines, neurotransmitters, or hormones[22,23], while immune-related alterations in the CNS may in turn originate from peripheral blood[10,24] (Figure 1).

Whilst there are some encouraging preliminary data to support the notion of peripheral biomarkers for Sz, it must be acknowledged that Sz is a complex and heterogeneous disorder[25,26] which needs to be further dissected into subtypes using biological based and clinical markers[27]. Therefore it is probable that some types of peripheral blood biomarkers may only define sub-sets of people with Sz. In addition, whilst most studies have focused on diagnosing Sz, biomarkers that can indicate the clinical course or drug treatment response will also be of clinical use. Another concern is that whilst the use of convergent functional genomics methods[12,28] is a good idea, so far the selection of most candidate biomarkers has been based on findings related to the brain. This approach would be likely to miss any periphery oriented dysregulations which could be associated with Sz. The scope of this review is to critically examine published studies of blood-based biomarkers for Sz, focusing on possible uses for diagnosis, drug treatment response, or their relationship with phenotypes associated with Sz in different blood cell types.

GENERAL DESCRIPTION OF THE INCLUDED STUDIES

Manuscripts were identified by searching PubMed (http://www.ncbi.nlm.nih.gov/pubmed) using the keywords "schizophrenia" and "peripheral" and "biomarker", in addition any articles whose title included "schizophrenia" and "peripheral" were also included. Only articles written in English and published between year 1995 and 2015 were included, the details of which are given in supplementary table. Briefly, of the 79 identified studies, 35% were performed in serum/plasma; 24% in peripheral blood mononuclear cells (PBMC) including monocytes and lymphocytes; 16% in lymphocytes; 13% in whole blood; 7% in white blood cells (leukocyte) including monocytes, granulocytes, and lymphocytes; 4% in platelets; and 1% in red blood cells (Figure 2A). In terms of experimental targets of analysis, nearly 40% examined differences in protein levels including cytokines and metabolites; 28% examined mRNA levels; with the remaining studies focused on enzyme activity assays (11%), DNA methylation patterns (7%), cell numbers (5%), miRNA levels (5%), or others (3%) (Figure 2B).

Overall, the studies can be sorted into categories which include: (1) brain-derived neurotrophic factor

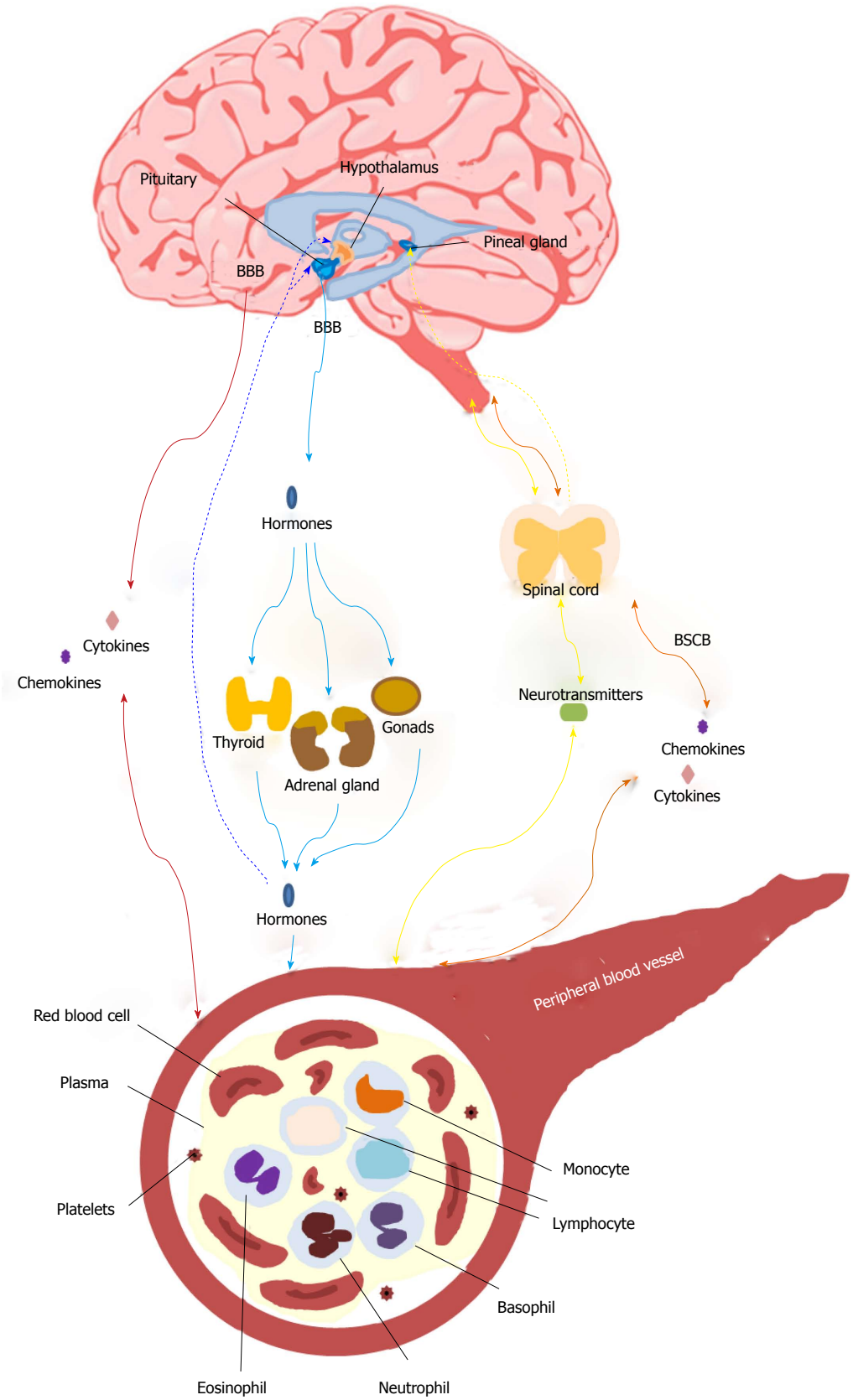

Figure 1 A schematic representation of central nervous system-peripheral blood tissue interactions. CNS stress may influence gene expression, DNA methylation, and cell metabolism in the peripheral blood *via* cytokines, neurotransmitters, or hormones with different transportation methods. Cytokines or chemokines can transport across the BBB (red lines) or BSCB (orange line) either from CNS to peripheral blood tissue or vice versa. The hormones are exerted by CNS and transported across the BBB *via* blood system to the target tissue (blue lines) and in turn regulate CNS through negative feedback (blue dashed lines). Another connection is the stimulated (yellow line) or negative feedback inhibition (yellow dashed line) *via* spinal cord *via* the parasympathetic or sympathetic nervous system. It is noted that there are several blood cell types with their own features in the peripheral blood vessel. The figure is an extension of Figure 1 in Marques-Deak *et al*[23]. BSCB: Blood-spinal cord barrier; CNS: Central nervous system; BBB: Blood-brain barrier.

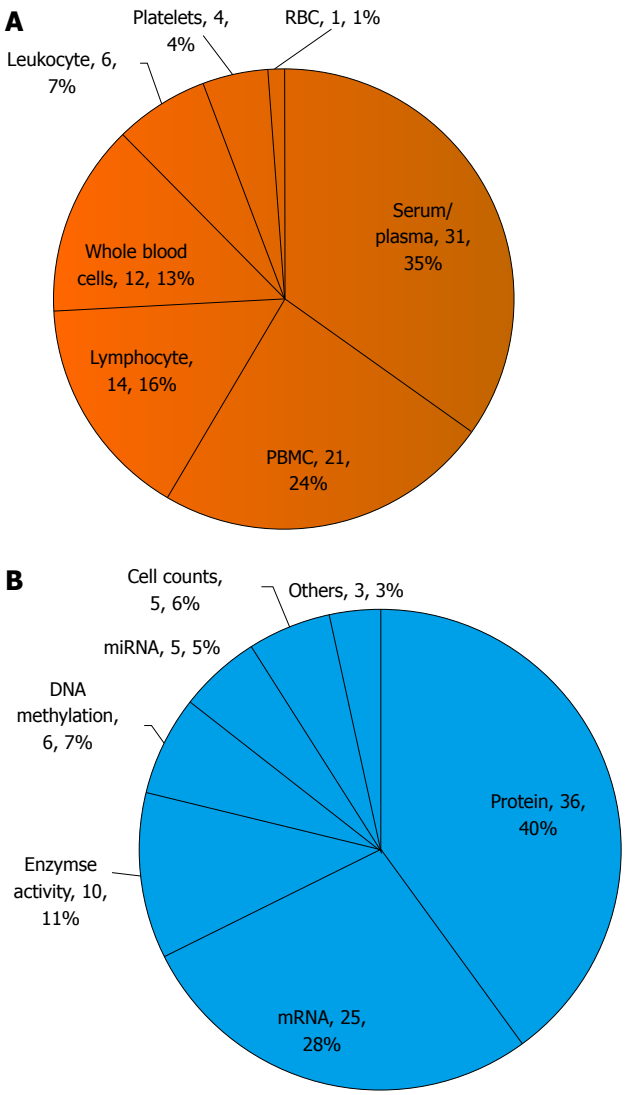

Figure 2 A summation of studies into peripheral biomarkers for schizophrenia. Studies are classified based on A: Blood cell types (*e.g.*, serum/plasma, PBMC, lymphocytes, whole blood cells, leukocyte, plateles or red blood cells); B: Research focus (*e.g.*, proteins, mRNA, enzyme activity assays, DNA methylation, or miRNA). The numbers indicate the number of studies in the categories, and its overall percentage. PBMC: Peripheral blood mononuclear cells; RBC: Red blood cell.

(BDNF); (2) molecules involved in inflammation and immune function; (3) neurochemistry; (4) oxidative stress response and metabolism; (5) epigenetic regulation and miRNA; and (6) transcriptome and proteome studies. In the following sections, we organized the narration based on biological functions and pathways while emphasizing (1) the biomarkers for diagnosis (D); (2) drug treatment response; (3) their correlations with phenotypes (*i.e.*, clinical symptom, cognitive function, or brain imaging) present in people with Sz.

BDNF

It has been postulated that Sz is associated with altered neurotrophin levels in blood, particularly lowered levels of BDNF[29,30]. A recent meta-analysis[30] of a total of 41 studies and more than 7000 participants indicated that the serum/plasma BDNF levels were moderately reduced in Sz compared with controls. Using DNA from whole blood, it has also been suggested that the promotor region of the *BDNF* gene has higher methylation levels in people with Sz[31] and this alteration may also be present in the brain[32]. Although the meta analysis showed that serum/plasma BDNF levels did not correlate with either the positive or negative symptom in Sz people[30], the plasma BDNF levels were positively correlated (r^2 = 0.14) with certain cognitive functions (*e.g.,* semantic generation tasks)[33] and auditory processing after computerized cognitive training[34]. Notwithstanding the significant result in Sz studies, plasma BDNF is unlikely to prove to be a specific biomarker for the disorder because reduced plasma BDNF is also present in bipolar disorder[35,36] and major depressive disorder[36,37].

Combining the study of peripheral blood levels and neuroimaging, it has been shown that BDNF levels in the serum/plasma positively correlate with N-acetylaspartate (r^2 = 0.15)[38], a marker of neuronal integrity, and brain activity (r^2 = 0.2)[39], which is measured by functional magnetic resonance imaging, in the parietal cortex. This means that peripheral BDNF levels may be a prognostic marker, as they return to normal during remission from acute states of bipolar disorder and major depressive disorder[36]. Whether people with Sz show similar patterns throughout different phases of their illness warrants further investigation.

The question of whether antipsychotic treatment itself can affect BDNF levels has resulted in conflicting data. A meta-analysis reported that antipsychotic treatment increased BDNF levels in plasma[30] but this was not seen in studies which examined levels in serum. However, it should be noted that the heterogeneity across studies in meta-analyses was high and the increase in BDNF levels after antipsychotic treatment was small (Hedges' g = 0.24). Furthermore, most of the studies evaluating changes of BDNF levels with antipsychotic treatment produced negative result for people with Sz[40-43] and those with treatment resistant Sz[44]. More detailed longitudinal studies will probably be required to determine if increased BDNF levels in the serum/plasma is a trait biomarker for the disorder or has some prognostic value as a disease state biomarker that can predict improvements in symptoms with treatment.

INFLAMMATION AND IMMUNE FUNCTION

Another category of biological pathways that has been receiving a lot of attention in the search for markers for Sz are those involved in immunological/inflammatory processes[9,10,45]. A comprehensive review[46] of serum/plasma biomarkers that allow the differentiation of people with Sz from controls suggests that more than 70% of potential biomarkers for the disorder are involved in the inflammatory response[46]. The

immune related biomarkers from the review include S100 calcium-binding protein B (S100B), interleukin 6 (IL-6), IL-2, and tumor necrosis factors (TNFs). The cytokines most frequently reported as changed in the serum of people with Sz include up-regulation of pro-inflammatory cytokines (*i.e.*, IL-6, IL-2, TNF, IL-1β and IL-8) or some of their receptors [*i.e.*, IL-2 receptor-α (IL-2Rα), IL-1 receptor antagonist[46,47]]. In addition, levels of mRNA of *TNF-α* and *IL-1β* genes have been shown to be increased in PBMC from people with Sz and their siblings, compared to controls[48], whereas, reduced interferon-γ (IFN-γ) mRNA has been identified in PBMC of people with Sz[49]. However, it should be noted that people with bipolar disorder also showed increases in some of the pro-inflammatory cytokines such as IL-6, TNF, and IL-1 in serum. Similarly, when a study examined the cell cycle-related histone biochemical properties (*i.e.*, total histone synthesis rates and H2A and H3 histone variant patterns of the nuclear extracted histone fraction) of lymphocytes, there are alterations that are common to both Sz and bipolar disorders[50]. Finally, in terms of cell numbers, T lymphocytes and monocytes have been found to be increased in Sz[51,52]. Taken together, these results indicate that people with Sz showed increases in pro-inflammatroy cytokines as well as lymphocyte numbers. However, some of these changes in "inflammation" components are shared with people who have bipolar disorder and this may reflect commonalities between these two disorders.

It is worth noting that, contrary to what occurs in people with Sz, a study investigating systemic inflammatory condition in a large cohort of individuals undergoing their first episode of psychosis (n = 117) showed that the majority of soluble elements of the pro-inflammatory are not significantly altered in PBMC[53]. However, they did find significant increases in intracellular molecules involved in pro-inflammatory pathways in PBMC (*i.e.*, nuclear factor κB, inhibitory complex IκB, inducible isoforms of nitric oxide synthase, cyclooxygenase) alongside significant decreases in some anti-inflammatory proteins (*i.e.*, prostaglandin 15-dexoy-PGJ_2 and peroxisome proliferator activated receptor-γ). The results indicate that the peripheral markers may change at different disease stages, but together suggest that the peripheral immune system is over-activated in both individuals undergoing their first episode of psychosis and people with Sz. However, it is important to note that the immune system is dynamic and will be sensitive to changes of cellular environment or medication status, which are confounds often not controlled for in the studies in Sz[10,45,46,54].

Evidences have shown that antipsychotic drugs can modulate components of the inflammatory-related pathways[55-57]. For example, serum levels of pro-inflammatory cytokines (*i.e.*, IL-2)[58,59] and anti-inflammatory cytokines (*i.e.,* IL-1RA and IL-10)[60] have been reported to be lower after treatment with typical or atypical antipsychotic drugs[58,59]. In lipopolysaccharides (LPS)-induced or polyinosinic: Polycytidylic acid-stimulated PBMC cultures, antipsychotic treatment altered immune function by suppressing the levels of IFN-γ and MCP-1 and raising the levels of IL-4, IL-10, IL-18 and RANTES[61,62]. Immune cell counts can also change in people with Sz after antipsychotic drug treatment; haloperidol increased CD3+ T cells, CD4+ T cells, and IL-2-secreting cells, together with CD4/CD8 ratio after 12-wk of treatment[63]. In addition, clozapine treatment initially (12 wk) increased CD34+ T cells, neutrophils and leukocytes. Over longer term, slightly elevated plasma IL-6 levels were observed in chronically clozapine monopharmacy-receiving patients, compared to controls[64]. Therefore, whilst it is still too early to draw any firm conclusions, current data indicate that decreased levels of cytokines are associated with antipsychotic treatment, whereas, T cells numbers were generally elevated by antipsychotic treatment.

The immune responses to changes of cellular environment have been measured and reported to be altered in people with Sz[62,65,66]. For example, out of 107 immune molecules, less IL-18 and more S100A12 (ENRAGE) were secreted by blood cells in *ex vivo* cultures obtained from first-onset people with Sz compared to controls following stimulation condition with a T cell stimulator (*i.e.*, anti-CD28/CD49d)[66], an effect replicated in an independent cohort[66]. In addition, T cells from people with Sz had significantly lower proliferative responses following stimulation with anti-CD3, compared to controls[65], a response not affected by antipsychotic medication. When PBMC from people with Sz were stimulated with bacterial LPS they released more protein of MCP-1, MIP-1α, IL-8 and IL-18 but less RANTES (for "regulated upon activation normal T cell expressed and secreted", a pro-inflammatory chemokine) and IFN-γ[62]. Collectively, the data suggests that changes of immune response to changes of cellular environment in Sz people could be complex.

There is evidence to suggest that the levels of some inflammatory molecules in blood are correlated with symptom severity in people with Sz[10,33,48,60,63,67]. For example, plasma levels of IL-2 in people with Sz were reported to be positively correlated with the scores in cognitive tests of digit span test (r^2 = 0.17) and intelligence (r^2 = 0.19), and negatively correlated with the presence of negative symptoms (r^2 = 0.2[67]), levels of IL-1β and TNF-α mRNA in PBMC were positively correlated (r^2 = 0.17) with scores on the general psychopathology factor of the PANSS[48] and changes in serum IL-10 levels were significantly correlated with improvements in symptoms [*i.e.*, negative, general psychopathology and total score (r^2 = 0.1-0.2)] among people with first-onset Sz[60]. Levels of the chemokine, CCL-11, were not only higher in people with Sz but were negatively correlated with performance on the working memory test (r^2 = 0.16) and positively correlated with a cognitive flexibility task (r^2 = 0.26[33]). Moreover, increased S100B positive natural killer cells in blood from people with acute Sz correlated with the perception of stress (r^2 = 0.08[68]) whilst increases in

CD4 positive cells correlated with an improvement of clinical symptoms in people with Sz (r^2 = 0.1)[63].

In summary, people with Sz demonstrate a distinct profile of immune peptides, aberrant immunological responses, and changes of immune cell counts. For example, higher plasma levels of IL-2 appears to be a promising Sz specific biomarker[47] which may reflect the clinical symptom as well as cognitive function[67] particularly since the levels decrease following treatment with antipsychotic drugs[58,59]. Some of these changes in the immune system markers correlate with treatment responses and symptom profiles, suggesting they may be suitable theranostic markers. However, the majority of the studies focused on cross-sectional group differences, and since the immune process is highly dynamic and changes are often transient, more research into the longitudinal patterns of immune profiles will be helpful to determine whether markers of this system are good candidates for indicating treatment responses.

NEUROCHEMISTRY

Monoamine pathways

Monoamine neurotransmitters such as dopamine (DA), norepinephrine (NE) or serotonin [5-hydroxytryptamine (5-HT)] have been postulated to associate with pathogenesis of Sz, primarily as a result of the pharmacological profiles of the drugs used to treat the disorder[69]. A number of dopaminergic markers have been evaluated in blood from people with Sz, including DA receptors (*i.e.,* DRD_2, DRD_3, and DRD_4[70]), DA transporter (DAT), and other molecules associated with the dopaminergic system [*i.e.,* tyrosine hydroxylase (TH)]. Both mRNA expression and receptor binding of DRD_2 were increased in lymphocytes from people with Sz who were drug-naive[71,72], however the up-regulation of DRD_2 mRNA was not replicated[73,74]. The levels of lymphocyte DRD_3 mRNA was reported to be elevated in both people with chronic Sz[73,75] and people with Sz who were drug-naïve[76]. However, it was also reported to be down-regulated in people with Sz and people with bipolar disorders[77]. For DRD_4, the mRNA has been reported to be either down-regulated in CD4 positive T cells[73] or not different in people with Sz[74]. It is noteworthy that higher DRD_3 mRNA has been reported in people with heroin addiction, whilst lower DRD_4 mRNA has been reported in people with major depression as well as during alcohol and heroin withdrawal[70].

There is evidence to suggest that lymphocyte DRD_2 mRNA levels positively correlate (r^2 = 0.2) with positive symptoms of Sz[74]. This provided some indirect support for the report that DA uptake by platelets correlated with delusional state of people with Sz[78]. In addition, the changes of lymphocyte DRD_3 and DRD_5 mRNA were associated with symptom severity (effect size = 1.46)[76].

As well as these changes in markers for DA receptors, DAT mRNA is reported to be higher in lymphocytes from people with chronic Sz compared to controls[74], by contrast, reduced DAT binding was reported in people with Sz[79], suggestive of a lower number of the DAT protein. TH mRNA has been reported as increased in PBMC of both people with Sz and their siblings compared to controls[48]. Other changes such as elevation of plasma homovanillic acid (HVA), a breakdown product of DA, were noted among people with Sz[80], people in the prodromal phase[81] and those with schizotypal personality disorder[82] but not in people with bipolar disorder[80], suggesting some disorder specificity. In addition, protein of DA- and cAMP-regulated neuronal phosphoprotein of 32 kDa (DARPP-32), a critical downstream target of DRD_1 and DRD_5-mediated signaling, was decreased in CD4 positive T lymphocytes and CD56 positive natural killer cells from people with Sz in comparison to control group[83], indicating lymphocytes may therefore function as an easily accessible model to study the DA intracellular signaling in the cells of people with Sz. Overall, some of the markers (*i.e.,* TH and HVA) may prove to be markers for a high risk population, such as people with a family history of the disorder as they were not only dysregulated in people with Sz[48,80] but also in their siblings[48] as well as in prodromal individuals of Sz[81] or people with schizotypal personality disorder[82].

One caveat of using monoamine related molecules as biomarkers is that most of the antipsychotics would block their receptors[69]. This effect is not limited to the CNS, therefore the drugs may dynamically change the peripheral profile of monoamine-related receptors and metabolites. Thus monoamine related molecules in the blood may prove to be state markers instead of the stable trait markers that are suitable for diagnosis. However, studies on the longitudinal changes in peripheral monoamine related molecules necessary to address this hypothesis are scarce. Thus, in terms of the effect of antipsychotics, a single study found that lymphocyte DRD_2 and platelet $5\text{-}HT_{2A}$ receptor binding were both reduced after treatment with antipsychotics[84]. In addition, lymphocyte DRD_3 and DRD_5 mRNA was reported to show dynamic, non-linear changes in people with SZ who were drug-naïve in the beginning of follow-up[76]. Briefly, mRNA of the DA receptors peaked at week 2 after taking antipsychotics, after which it decreased but was above baseline at week 8[76,77]. Finally, it has been reported that both risperidone and clozapine elevate plasma NE levels, with risperidone producing a smaller effect[85]. Logically, considering their close ties with the pharmacological properties of antipsychotics, peripheral monoamine related molecules might be a good indicator for treatment response but current evidence to support this posit is lacking.

Glutamatergic pathways

Because glutamatergic dysregualtion may play a role in the pathogenesis of Sz[69], there is a body of research focusing on peripheral levels or functions of the amino acids, such as glutamate, D-serine, L-serine, glycine, and agmatine, that activate N-methyl-D-aspartate

(NMDA) glutamate receptors[86-89] and are involved in modulating the glutamatergic neuronal pathways.

Most studies focused on peripheral glutamate levels in people with Sz. In a meta-analysis[86] of 10 studies, higher levels (standardized mean difference = 0.64; 95%CI = 0.21-1.06) of glutamate have been reported in blood from people with Sz compared to controls, however, similar dysregulations have been reported in the small number of studies that focused on major depressive disorder[90,91], suggesting the alteration of glutamate levels is not specific to the diagnosis of Sz. Interestingly, the response of platelet intracellular calcium to glutamate was greater in people with Sz than in controls, suggesting NMDA receptor may be more sensitive in these people[87]. A single study has reported that people with Sz had higher levels of plasma agmatine, an endogenous substance that is synthesized from L-arginine and is proposed to be a new neurotransmitter, than controls[88], and that the levels correlated with their PANSS score, suggesting that plasma agmatine levels may be a potential diagnostic biomarker for Sz. Lower plasma D-serine, D-/L-serine ratio were found in people with Sz who were treatment-resistant[89], but chronically people with Sz showed higher plasma D-serine and glycine levels[92]. In addition, the plasma levels of D-serine were associated with improvements in positive symptom[92], but not with cognitive functions in people with Sz[93]. A study examining the effects of clozapine treatment on D-serine, L-serine, and glycine in people with Sz who were treatment-resistant, showed glycine levels and the glycine/L-serine ratio were significantly increased following clozapine treatment, suggesting that plasma L-serine and glycine could be potential therapeutic markers[89]. In summary, higher levels of blood glutamate has some potential to be a diagnostic marker for Sz, but its correlation with major depressive disorder warrants further investigation in order to determine the specificity of the changes.

Other neurochemicals

Of other neuronal chemicals associated with the pathogenesis of Sz, levels of plasma γ-aminobutyric acid have been reported to be lower in people with Sz as well as bipolar disorders compared to the control group[80]. By contrast, plasma reelin[94], which is involved in the migration of neurons; and adenosine deaminase activity[95], a homeostatic modulator that affects brain DA and glutamate activities, were higher in people with Sz. It is also of note that acute metabolic stress in the brain, induced by pharmacological doses of 2-deoxyglucose (2DG), a glucoprivic agent transported across the blood-brain barrier into brain tissue where it inhibits intracellular glucose metabolism and produces a clinical state similar to hypoglycaemia[96], appears to affect the peripheral vesopressinergic system *via* the pituitary-adrenal axis[96,97]. In addition, after 2DG treatment people with Sz had higher 2DG-induced plasma adrenocorticotropic hormone levels[97], HVA, and 5-hydroxyindoleacetic acid levels[96]. These data suggest that central acute metabolic stress may alter the metabolism in peripheral dopaminergic and serotonergic systems *via* hormones (Figure 1). In PBMC, both people with first episode of psychosis[98] and chronic Sz[99] show decreased protein levels of cannabinoid receptor 2 (CB2), however, levels of CB2 were influenced by the medications and cannabis use history[98], suggesting endocannabinoid system presented at the initial phases of psychosis could be contributing to the pathophysiology of the disease and constitutes a possible treatment response biomarker of psychotic disorders[98]. In the cholinergic system, a decrease of a7 acetylcholine receptor (AChR) mRNA levels has been reported in lymphocytes from people with Sz[100] that was not affect by antipsychotic treatment, suggesting AChR as a peripheral trait makers of Sz. Finally, a single study reported that the density of the peripheral-type benzodiazepine receptors in platelets is a predictor for aggressive behaviors in people with Sz[101] showing an association with higher scores for overt aggression, hostility and anxiety. This particular marker may prove useful for defining a subgroup within the syndrome of Sz. However, considering limited evidence so far, it is too early to predict the robustness of these markers. All of the markers require validation in larger cohorts and need to be assessed for specificity before their usefulness can be assessed.

OXIDATIVE STRESS RESPONSE AND METABOLISM

The pattern of oxidative stress markers in the plasma was examined in several studies. Psychotic patients in general are in a significantly increased long-term condition of oxidative stress[102,103]. Oxidative stress markers, such as pentosidine[104], glycer-AGE[105] and thiobarbituric acid reactive substances[103,106] were found to be higher among people with Sz and carbonyl stress was found to be lower in people with the disorder[104,105,107]. However, those oxidative markers have overlapping levels between people with Sz and healthy individuals, suggesting the discriminability of those markers is low[102]. One group suggested that only a subgroup of study participants with the glyoxalase Ⅰ (GLO1) deficits showed distinct patterns of oxidative stress abnormality in the plasma[104], suggesting GLO1 deficits and oxidative stress may contribute to development of a certain subtype of Sz. However, evidence also suggests these oxidative markers show dynamic non-linear changes during hospitalization and levels could be associated with the dosage of antipsychotics irrespective of the treatment responses[107]. If this finding is replicated, the utility of these markers may be restricted to determining medication compliance.

In light of the tendency of people with psychiatric disorders, particularly Sz, to develop metabolic syn-

drome, several studies have examined molecules involved in glucose homeostasis[95,108-113]. In a study where PBMC were stimulated *ex vivo* with staphylococcal enterotoxin B, eight out of total of 18 proteins examined were differentially expressed in cells from people with Sz who were first onset and drug-naive Sz compared to controls. These proteins belonged to the glycolytic pathway[110], indicating that impaired glycolytic response could be a potential early stage of diagnostic biomarker for Sz. Mitochondrial complex Ⅰ, the first enzyme in the mitochondrial respiratory chain for the oxidation of glucose, was found to have increased activity in the platelets from people with Sz[108] and to have higher mRNA levels in blood from people with Sz[111,113]. It has also been reported that the activity of platelet mitochondrial complex Ⅰ is associated with positive symptoms and clinical disease course in people with Sz[108]. Significantly, the activity of platelet mitochondrial complex Ⅰ is not altered in people with bipolar disorder[106] and mRNA levels for platelet mitochondrial complex Ⅰ is not altered in white blood cells from people with autism spectrum disorder[113]. These latter findings are an indicator that the activity of platelet mitochondrial complex Ⅰ is associated with positive symptoms and clinical disease course may be specific to Sz and a potentially useful biomarker for those features of the disorder.

The utility of these markers is thrown into doubt by a study in T-lymphocytes which reported that antipsychotic drugs affect the expression of a large number of genes and that some of those genes were related to oxidative stress and metabolic disease[114]. In addition, it has been suggested that molecules involved in glucose homeostatsis may also be predictors for people who develop extrapyramidal symptoms after receiving antipsychotics[115]. Thus, it may be that rather than being markers for diagnosis, some of the molecules involved in glucose homeostatsis might be potential markers for either susceptibility to side-effects or for medication compliance.

EPIGENETICS AND MICRORNA

Epigenetic factors can potentially alter susceptibility to Sz[116]. Thus, it is not suprising that studies have evaluated the global methylation pattern in people with Sz[117-120]. Overall, leukocyte DNA from people with Sz had lower methylated deoxycytidine (mC) content compared to controls[120]. In addition, a genome-wide study showed there were 234 CpG sites (0.04% of CpG sites) that were differentially methylated in both people with Sz who were drug-naïve compared to controls and in people with Sz compared to their discordant monozygotic twin sibling[118], with most of the sites located in promoter regions of genes. Further studies have reported that in PBMC from people during their first onset of Sz there were 603 CpG sites (2% of CpG sites) that were differentially methylated[119], the genes that were particularly affected were related to the nuclear lumen, transcription factor binding, and nucleotide binding. Using whole blood, a methylome-wide association study showed that 139 CpG sites (0.003% of CpG sites involved in analyses) were differently methylated in people with Sz, highly implicating an exon and 3'UTR of FAM63B and an intron of RELN[117]. Overall, differentially methylated CpG sites can be detected in peripheral blood of people with Sz and many of the methylation was reported in the promoter regions of genes, therefore they may affect transcription of these genes. However, it remains to be determined if the changes in methylation are correlated to changes in levels of gene expression, the mechanism by which methylation predominantly regulates gene activity.

The central regulatory role of microRNA (miRNA) in gene expression[121] makes them promising biomarkers for Sz. Whilst there have been a number of studies focusing on miRNA in brain samples[122], relatively few studies have examined them in peripheral blood. Of these studies, reported changes in miRNA include up-regulation of hsa-miR-34a[123-125] and down-regulation of hsa-miR-432[123,126] in PBMC from people with Sz, which have been replicated in independent studies[125]. However, the data for other miRNAs are conflicting. For example, hsa-miR-21 and hsa-miR-30e have been reported to up-regulated[125,127] and down-regulated[126] in PBMC from people with Sz. Likewise, levels of hsa-miR-30e and hsa-miR-181b were altered in opposite directions[124-126] in studies using plasma from people with Sz. It has been reported that antipsychotic treatment is associated with changes of miRNA levels in human plasma[124,128] and T-lymphocyte cell lines[114]. The expression levels of hsa-miR-365, hsa-miR-520c-3p, and hsa-miR-181b in plasma were significantly down-regulated after antipsychotic treatment[124,128], of these, miRNA-181b expression levels were positively correlated with the improvement of negative symptoms[124]. Finally, an *in vitro* antipsychotic treatment response study in T-lymphocyte cell lines demonstrated that miR-200c-3p and miR-28-5p were down-regulated in haloperidol-treated cell lines compared to controls[114]. Given the variance in data regarding levels of peripheral miRNAs, the consistent findings of hsa-miR-34a and hsa-miR-432 as well as their expressions levels were not changed following treatment, these have implied their potential as diagnostic markers.

In sum, peripheral epigenetic markers might be a promising direction for finding biomarkers for Sz. The methylation patterns and miRNA expression patterns have both shown differential patterns between people with Sz and controls. However, considering the discrepant results mentioned above, the consistency between studies warrant further investigation.

THE HUMAN TRANSCRIPTOME AND HUMAN PROTEOME STUDIES

Modern technologies allow the investigation of levels of

thousands of mRNAs and proteins at the same time. The studies at the levels of mRNA are known as studies of the transcriptome whereas studies at the levels of proteins are known as studies of the proteome.

Most studies of the transcriptome have involved measuring levels of mRNA is gene expression microarrays. In psychiatry, such studies have involved the use of whole blood[129-131], lymphocytes[132] or PBMC samples[133-135] to identify potential biomarkers for Sz. Other studies examined proteomics information in RBC[109], T cells[136], and serum[61,109,137,138] in attempts to answer the same question. This approach has typically led to a large set of mRNAs or proteins being proposed as possible biomarkers in Sz but most findings await replication.

Briefly, in a whole blood study from people during their first onset of Sz, the expression of glucose transporter (SLC2A3) and actin assembly factor (DAAM2) were increased, whereas zinc metallopeptidase, neurolysin 1 and myosin C were significantly decreased, compared to controls[129], indicating peripheral mRNA of these genes could be a potential biomarkers in early stage of disease course. A genome-wide expression analysis in PBMC samples from people with Sz was characterized by alterations of genes with immune system function[133,135] with some differentially expressed genes, such as argonaure 2, myocyte enhancer factor 2D, Enah/Vasp-like, peptidase inhibitor 3, S100 calcium binding protein A12 (S100A12), defensin α4 (DEFα4[133]) as well as AKT1[135] being confirmed as changed using qPCR, the result supporting the hypothesis that the disorder has a significant immunological component in its etiology. In addition, a microarray study identified six genes [including B cell translocation gene 1, glycogen synthase kinase 3α (GSK3α), HLA-DRB1, heterogeneous nuclear ribonucleoprotein A3, selenium-binding protein 1 (SELENBP1), and splicing factor] which were differentially expressed in both brain and peripheral blood of people with Sz[13]. Of these, SELENBP1 was identified as the strongest candidate biomarker among all genes differentially expressed in Sz, because it was the only gene showing significant differential expression in a similar direction in both brain and blood[13]. However, the result of up-regulation of SELENBP1[139] and up-regulation of GSK3A[112] in peripheral blood was not replicated in the other studies. Another study reported several genes related to nucleosome and histone structure were dysregulated in PBMC of both people with Sz and their siblings, suggesting a potential epigenetic mechanism underlying the risk state for the disorder[134]. Other differentially expressed genes in PBMC were involved in pathways such as cell adhesion and neuronal guidance, neurotrophins, oxidative stress and glucose metabolism, and apoptosis and cell-cycle regulation[135], all of which have some face value for the pathophysiology associated with Sz.

For the studies reporting discriminability of the markers, a study using whole blood from people with Sz who were drug naïve identified 14 probes which annotated 11 genes (listed in supplementary material) that predicted diagnosis with 91.2% accuracy in the training set and 87.9% accuracy in the validation set[131], however, this study lacked qPCR analysis to validate the microarray data. A microarray using white blood cells of discordant sib-pairs for either Sz or bipolar disorder identified a set of 35 transcripts which can discriminate people with Sz from people with bipolar disorders and their unaffected sib-pairs with 95% accuracy[130]. However, only few genes [*i.e.*, Serotonin receptor type 4 (5HT4), transcription factor-like 4, and neuregulin 1] were confirmed using qPCR and there has been no subsequent validation study for the prediction model. Collectively, the inconsistent findings of this type of study may result from the heterogeneity of the preparations used, the people with Sz being at different clinical courses of the disorder (*i.e.,* first onset or chronic stage) and the fact that Sz is a spectrum rather than a discrete illness.

Of the studies discussed, only one followed up the differentially expressed RNA levels in whole blood of first onset Sz people for 12-24 mo[129]. DAAM2, one of five differentially expressed genes, returned to control levels in the whole blood when people were in remission after their first psychotic episode, suggesting this mRNA could be altered by antipsychotics and may be a potential marker for treatment response. Taken together, there is only down-regulation of intercellular adhesion molecule in people with Sz that were replicated in at least two microarray studies[138,140]. Other studies have identified markers that may be of use in identifying people who are at risk of developing the disorder, such as the up-regulation of DEFA in T cells and plasma from people with Sz, and both their affected or unaffected monozygotic twin[136]. Of interest is the down-regulation of apolipoprotein A1 identified in both red blood cells of people with Sz and serum of people with Sz who were drug-naive[109]. Not only does this suggest it might be a trait marker but a similar pattern was shown in postmortem brain tissue[109], suggesting that the peripheral changes reflect central pathophysiology.

Proteomics studies have resulted in a signature comprised of 34 serum protein analytes that have 60%-75% discriminant accuracy in separating people with Sz from those with major depression, bipolar disorder, Asperger syndrome and controls[140], suggesting these proteins in blood serum as a potential biomarker for Sz. In addition, 27 serum proteins were identified as discriminating between people with Sz and healthy controls[138] and highlighted some proteins among them with important roles in the immune system. It has been reported that dysregulation of 4 serum analytes [up-refulation of 2-piperidinec carboxylic acid, along with the down-regulation of 6-deoxy-mannofuranose, galactoseoxime, and a serum peptide (m/z 3177)] have the best discriminating value between people with Sz, and controls[141], indicating that metabolomics and proteomic approaches can be used in the biomarker research.

Overall, the outcome of current omic studies has

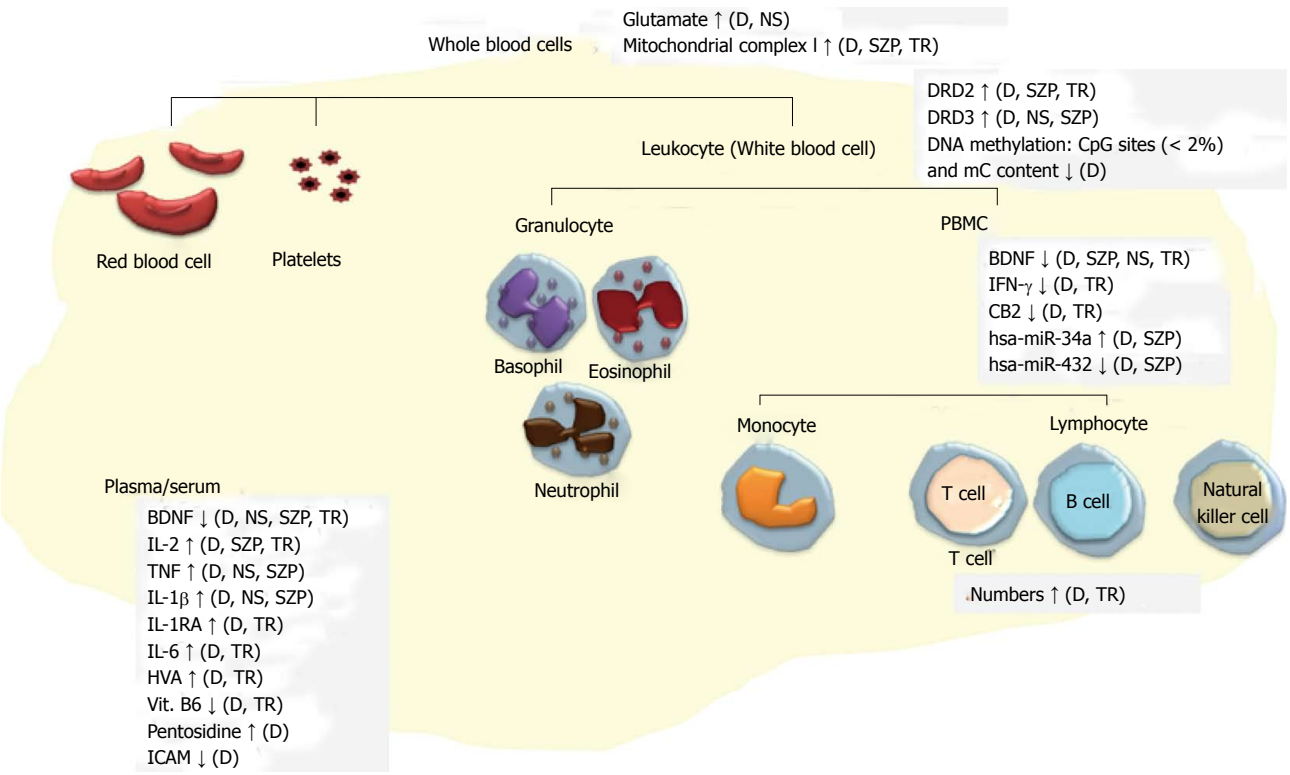

Figure 3 Representation of potential peripheral biomarkers for schizophrenia. Only markers consistently reported in two or more individual peripheral studies are listed in the figure. The markers were classified as either diagnostic (D), antipsychotic TR or both. SZP indicates those markers have been reported to correlate with phenotypes (*i.e.*, clinical symptom or cognitive function) in people with schizophrenia. Some markers were NS for schizophrenia. PBMC: Peripheral blood mononuclear cell; BDNF: Brain-derived neurotrophic factor; IL: Interleukin; TNF: Tumor necrosis factor; HVA: Homovanillic acid; Vit. B6: Vitamin B6 (pyridoxamine); ICAM: Intercellular adhesion molecule; DRD_2: Dopamine receptors D_2; IFN: Interferon; CB2: Cannabinoid receptor; TR: Treatment response; NS: Not specific.

been the identification of a number of potential biomarkers in peripheral blood with a cluster of reports suggesting that markers associated with immune function/inflammation might be of interest, however the utility of these need to be confirmed.

CONCLUSION

As can be seen from the précis of studies to date, the search for biomarkers of Sz in peripheral samples is flourishing. Markers that have consistently been altered in two or more individual studies are summarized in Figure 3. It is possible that some of the differential patterns reported to exist in people with Sz can be used for diagnostic purposes. However, only a few studies reported the sensitivity and specificity of the discriminability of their markers[108,123,125,130,138,140,141], leaving doubt as to the potential of the other molecules to act as markers. To try and assess the utility of these disparate markers, performing a meta-analysis based on the categories we used here might be helpful to identify potential markers that have large and consistent effect size across studies. On the other hand, as some studies have shown, peripheral biomarkers may be more useful as state indicators. The changes of some biomarkers appear to be associated with clinical outcome, treatment responses, and the changes of clinical symptoms, suggesting they might be useful for specific clinical events, an opportunity that has been ignored in the search for diagnostic markers.

An important factor that hasn't been considered in many studies is whether or not the candidate peripheral biomarkers showed similar patterns in other psychiatric diseases. It is important to recognize that several psychiatric diseases can share some of the same components with Sz[12,142,143]. Based on the limited studies so far, many of identified markers were not specific to Sz but were shared with other psychiatric diseases such as bipolar disorders[47,50,70,80]. The next step of peripheral biomarker development would be examining the candidate markers in other psychiatric diseases. Having Sz specific biomarkers can increase the efficiency of disease diagnosis as well as improving treatment strategies.

One question raised by this review is the blood-brain relationship. Most of the studies mentioned above (approximately 70%) were prompted by findings related to the brain. For instance, neurotransmitters apparently behave differently in the peripheral system than in the brain, yet many studies have focused on the neurotransmitters implicated from neuroscientific studies of Sz. This often leads to inconsistency among studies, and patterns found in the brain oftentimes fail to be found in the peripheral blood. Nevertheless, even if the neurotransmitters were to behave differently in the periphery, some evidence suggests they can

be biomarkers for treatment effect. We suggest the biomarkers in the peripheral blood should be initially simply viewed as whether or not they have clinical utility rather than whether they may be tied to the aetiology of Sz. If a similar dysregulation can be seen in the brain or the aberration of the peripheral molecules were related to clinical symptoms it would add more information for interpreting the potential peripheral biomarker. In this respect, given it is known that many biomarkers have been shown to be affected by factors such as fasted/fed state, diurnal and seasonal rhythm and disturbances in sleep[57] efforts should develop standardized blood collection procedures in order to decrease study to study variation. Alternatively, the exact specimen collection protocol, time of collection, anticoagulants used and methods of blood processing should be accurately described in any blood study seeking to report findings on potential biomarkers in Sz.

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ORIGINAL ARTICLE

Retrospective Study

How does a real-world child psychiatric clinic diagnose and treat attention deficit hyperactivity disorder?

Kumi Yuki, Jyoti Bhagia, David Mrazek, Peter S Jensen

Kumi Yuki, Peter S Jensen, REACH Institute, New York, NY 10018, United States

Jyoti Bhagia, David Mrazek, Division of Child and Adolescent Psychiatry, Department of Psychiatry and Psychology, Mayo Clinic, Rochester, MN 55905, United States

Peter S Jensen, Division of Child and Adolescent Psychiatry, Department of Psychiatry, University of Arkansas for Medical Sciences, Little Rock, AR 72205, United States

Author contributions: Yuki K designed and performed the research, collected and analyzed the data, and wrote the paper; Bhagia J provided clinical advices for the research; Mrazek D designed the research and provided an initial supervision on the research; Jensen PS supervised the research design and the research process, analyzed the data, and revised the paper.

Institutional review board statement: This naturalistic study is a part of the original study entitled "Pharmacogenetics and the Care of Child and Adolescent Patients", which was reviewed and approved by the Mayo Clinic Institutional Review Board (IRB).

Informed consent statement: Because this study is a chart review and the research is exempt, a waiver of informed consent was allowed. Study subjects have given authorization for use of their Mayo Clinic electronic medical record for research purposes.

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Correspondence to: Kumi Yuki, MD, Research Scientist, REACH Institute, 485 7th Ave #1510, New York, NY 10018, United States. kumiyuki1026@gmail.com
Telephone: +1-507-3161166

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Abstract

AIM: To investigate child and adolescent psychiatrists' (CAPs) attention deficit hyperactivity disorder (ADHD) and oppositional defiant disorder (ODD) diagnoses and treatments in real-world clinical practice.

METHODS: The medical records of 69 ADHD children (mean age = 9.5 years), newly referred to the ADHD clinic, were reviewed for their scores of parent- and teacher-reported Vanderbilt ADHD Diagnostic Rating Scales (VADRSs), CAPs' diagnoses of ADHD and ODD, and CAPs' treatment recommendations. Among 63 ADHD subjects who completed both parent and teacher VADRSs, we examined the agreement of the parent and teacher VADRSs. We also examined the concurrent validity of CAPs' ODD diagnoses against the results from the VADRSs. In addition, we compared CAPs' treatment recommendations against established ADHD and ODD guidelines.

RESULTS: Among 63 ADHD subjects, the majority of the subjects (92%) met full ADHD diagnostic criteria at least in one setting (parent or teacher) on the VADRSs. Nearly half of the patients met full ADHD diagnostic criteria in two settings (parent and teacher). Relatively low agreement between the parent and teacher VADRSs were found (95%CI: -0.33 to 0.14). For 29 children who scored positive for ODD on the rating scales, CAPs confirmed the ODD diagnosis in only 12 of these case-positives, which is considered as a fair agreement between CAPs and VADRSs (95%CI: 0.10-0.53). For 27 children with no ODD diagnosis made by either CAP or VADRS, more than half of them were recommended for medication only. In contrast, where CAPs made the diagnosis of ODD, or where the parent or teacher VADRS was positive for ODD, almost all of the patients received recommendations for medication and behavior therapy.

CONCLUSION: CAPs' ADHD diagnoses have strong concurrent validity against valid rating scales, but ADHD's most common comorbid condition - ODD - may be under-recognized.

Key words: Attention deficit hyperactivity disorder; Oppositional defiant disorder; Vanderbilt attention deficit hyperactivity disorder Diagnostic Rating Scale; Quality assessment; Clinical practice

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Core tip: Given the concerns about possible attention deficit hyperactivity disorder (ADHD) over-diagnosis and over-treatment, within a newly diagnosed sample of consecutive ADHD patients, we examined the concurrent validity of child and adolescent psychiatrists' (CAPs) ADHD and oppositional defiant disorder (ODD) diagnoses against the results from the Vanderbilt ADHD Diagnostic Rating Scales. We also evaluated CAPs' ADHD and ODD treatment recommendations and discussed clinical implementations of the established treatment guidelines into CAPs' practice. In our samples, CAPs diagnosed ADHD strongly agreeing with the rating scales, but given our results showing the relatively low prevalence rates of ODD diagnosis within ADHD, ODD may be under-recognized.

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INTRODUCTION

In view of national concerns about the rising rate of diagnoses of attention deficit hyperactivity disorder (ADHD), increased understanding of the diagnostic procedures and accuracy of clinicians' diagnoses of ADHD and its associated conditions is needed[1,2]. Although diagnostic criteria for ADHD and other psychiatric disorders are clearly specified in the DSM-5; Diagnostic and Statistical Manual of Mental Disorders 5th ed[3], it is less clear whether clinicians use these criteria in clinical practices. Relatedly, ADHD frequently occurs with comorbid conditions, particularly oppositional defiant disorder (ODD), its most common comorbidity with 54% to 84% of prevalence in ADHD patients reported in the American Academy of Child and Adolescent Psychiatry (AACAP) practice parameter[4,5]. However, relatively lower prevalence of ODD in ADHD children has been reported in different samples most likely due to heterogeneity of outcome measures[6]. Given the greater risks for substance use, academic disability, and social dysfunction in ADHD children comorbid with ODD[7,8], identification and accurate diagnosis of ODD in the early stages is essential, to improve the prognosis[5,9,10].

However, implementing these guidelines in real-world clinical practice can be a time-consuming task[4,5,10,11]. To make the process more practical and feasible, many rating scales comprised of the DSM diagnostic criteria have been published as valid assessment tools for both ADHD and ODD[5,10]. Among many ADHD rating scales, the Vanderbilt ADHD Diagnostic Rating Scale (VADRS), published in 2002 by the AAP and National Institute for Children's Healthcare Quality, was designed to capture standardized ADHD symptom information from parents and teachers reporting on children's behaviors[12]. This rating scale was also designed to assist providers in screening ODD and other common comorbidities[13,14].

Furthermore, the parent VADRS has shown high concurrent validity with ADHD diagnoses (γ = 0.79) made *via* structured diagnostic interviews (C-DISC-IV) in elementary school children[12,15]. Recently, Wolraich *et al*[12] evaluated the validities of parent and teacher VADRS in a larger sample of children and concluded that the agreement between the teacher VADRS's symptoms and DISC-IV was statistically significant ($P < 0.05$; inattention γ = 0.33, hyperactive γ = 0.29), but of less magnitude of agreement compared to the parent VADRS with the DISC-IV[16,17]. The parent and teacher VADRSs have also been evaluated for their utility in assessing comorbid conditions such as ODD/CD and learning disorders[13,14]. Similarly, Becker *et al*[13] noted that a total score > 10 of the 8 ODD items on the parent VADRS demonstrated high sensitivity and specificity (0.88, 0.85 respectively) against C-DISC-IV diagnoses, with 91% of true positive cases identified.

The AAP and AACAP guidelines for ADHD[4,5] recommend that ADHD diagnoses be made *via* an intensive process that requires a careful diagnostic interview and review of other records (*e.g.*, school evaluations, other medical reports, rating scales, *etc.*). Ultimately, the physician's "best clinical judgment" must integrate all this information to render the final diagnoses. Despite

of the importance of the physician's "best clinical judgment" final step, little research has examined clinicians' best judgments in diagnosing ADHD and ODD[7,8,18], comparing clinicians' diagnostic judgments with the results from parent and teacher rating scales with a validated measure such as the VADRS.

Assuming that the correct diagnosis has been rendered, additional questions arise: Do physicians' recommendations follow treatment guidelines in clinical practice of ADHD? While ADHD guidelines generally recommend medication as a first-line treatment, combining medication with behavior therapy is frequently recommended and is the best option for most cases. Behavior therapy is a first-line intervention for ODD, which is also advisable as an initial approach for some ADHD cases, such as younger children with ADHD. Behavior therapy promotes positive parenting and provides an opportunity for social training for disruptive children, which may not be achieved by medication treatment only[2,19-24].

Given previous research showing the high agreement of specific VADRS thresholds against DISC-derived diagnoses[13,16,17], we hypothesized that physicians' clinical diagnoses of ADHD and ODD might be evaluated by comparing the results of the parent and/or teacher VADRSs with clinicians' final diagnosis of these conditions. We also reasoned that we could evaluate physicians' treatment recommendations by comparing them with the treatment guidelines for ADHD and ODD.

MATERIALS AND METHODS

Sampling frame

From June 1 to November 30, 2011, all records of pediatric patients newly referred to the ADHD Clinic at the Mayo Clinic in Rochester, Minnesota for initial evaluation were identified (n = 120, age range 3-18, and mean 9.4 years). Of these, 69 patients were selected through the inclusion and exclusion criteria described below.

Inclusion criteria

Patients, living in the immediate geographic area, who received their routine care either within the Mayo Clinic or its larger regional system, Mayo Clinic Health System, were included. Across the system, electronic medical records were available for examination by the study team. Thus, to be eligible, patients must have had an initial evaluation by CAPs within the ADHD clinic, and received their follow-up care either within the ADHD clinic or in the surrounding Mayo primary care settings.

Exclusion criteria

All patients who were followed up by non-Mayo Clinic providers were excluded (40 subjects). All patients not receiving an ADHD diagnosis after clinical evaluation were excluded as this study was a part of the naturalistic study to assess clinical implementations of VADRSs for diagnoses and treatment outcomes in ADHD children in the ADHD clinic. No comorbid conditions were used as exclusion criteria.

Records abstraction

Eight months after their initial diagnostic visit, patients' medical records were reviewed in order to gather diagnostic and treatment-related data, *i.e.*, ADHD and comorbid diagnoses, medications, therapies, and VADRS scores.

Sample characteristics

The mean age of the 69 children identified with ADHD was 9.5 years (age range, 4-18). As expected, males predominated, with a male/female ratio of 2.8:1. Eighty-two percent were Caucasians, 6% were African-American, 3% were Asian, and 6% were another ethnicity. Expected proportions of the various ADHD subtypes were found (combined subtype 41%, inattentive subtype 32%), except that nearly one-quarter of children were diagnosed with ADHD - not otherwise specified.

VADRS

As a part of its standard of care, the Parent and Teacher VADRS were used to assist in the assessment of ADHD symptoms and comorbid conditions at initial visits to the ADHD clinic. Among 69 patients, all of them had a completed parent VADRS at the initial visit, except for two incomplete ODD ratings. Six patients did not have teacher VADRS available for scoring at the initial visit. Among 63 subjects who had both parent and teacher VADRS completed, 59 subjects had completed parent and teacher ODD rating scales available for scoring. Accepted procedures for scoring the VADRS were used[25] as follows: On the VADRS, all ADHD and ODD symptoms were rated on a 0 to 3 scale (0 = none, 1 = minimal, 2 = often, 3 = very often). For any symptom to be considered "positive" for diagnostic purposes, it must be scored at a 2 or 3 (often, very often). When scoring the parent and teacher VADRS for diagnostic purposes, each of the two ADHD symptom subtypes (inattentive and hyperactive-impulsive) is considered to be screen-positive if > 6 of 9 of their respective symptoms are scored at 2 or 3. Additionally, the ADHD and ODD scores in each rating scale were added up to calculate the total ADHD and ODD scores (range of total possible ADHD symptom scores: 0-54; range of possible ODD symptom scores: 0-24 Parent VADRS, 0-30 Teacher VADRS). We applied the cut point of a total score > 10 of the eight ODD items on the parent VADRS considering its high sensitivity and specificity for a screening of ODD. However, for teacher VADRS, ODD is considered to be screen-positive when > 3 of 10 of their ODD symptoms are scored at 2 or 3 on the teacher VADRS.

Additional items on the VADRS also ascertain the presence or absence of impairment in functioning, another pre-requisite before making any ADHD or ODD diagnoses. Both the parent and teacher VADRSs have

Table 1 Comparison of vanderbilt attention deficit hyperactivity disorders Diagnostic Rating Scales parent *vs* teacher results *n* (%)

		Parent vanderbilt rating scale		Total
		Negative	Positive	
Teacher Vanderbilt Rating scale	Negative	5 (8)	17 (27)	22 (35)
	Positive	13 (21)	28 (44)	41 (65)
Total		18 (29)	45 (71)	63 (100)

eight items to assess children's performance levels, which are rated on a 1 to 5 scale (1 = excellent, 2 = above average, 3 = average, 4 = somewhat of a problem, 5 = problematic). The performance domains in the parent rating scale range from academic performance of reading, writing, and mathematics to social skills by assessing relationship qualities with parents, siblings, and peers as well as behavioral skills to attend organized activities. The performance domains in the teacher rating scale similarly include academic performance of reading, mathematics, and written expression, as well as social skills in terms of relationships with peers, but also behavioral skills such as following directions, not disrupting class, assignment completion and organizational skills. To consider the performance score as a functional impairment either at home or school, at least one item among 8 items must be scored at a 4 or 5 (somewhat of a problem, problematic).

In summary, we considered it as a positive diagnostic result when either parent or teacher's VADRS was positive (> 6 of 9 on either or both of the inattentive and hyperactive-impulsive subscales), along with the presence of impairment. For an ODD screening, either a total ODD score > 10 on parent VADRS or > 3 of 10 ODD items scored at 2 or 3 on teacher VADRS.

Clinical process to diagnose ADHD and ODD

As a part of the initial assessments, comprehensive assessments were conducted by board-certified child and adolescent psychiatrists (CAPs), capturing information from the child's guardians pertaining to the child's growth and development, medical and psychosocial history, school history and performance, test scores, and mental status examinations, as well as semi-structured diagnostic interviews to the parents for ADHD and related conditions, using DSM-IV criteria. The CAPs were free to examine the results of both the parents and teachers VADRS, which were collected not only at the initial evaluation but throughout the 8-mo period to track the child's symptoms and treatment response. No steps were taken to require CAPs to include the VADRS results as a part of their final diagnostic considerations, however.

Analytic approach

For all subjects, we determined the number and proportion of them who met criteria for ADHD and ODD in either a parent or teacher rating scale as well as in both of the scales, based on the recognized scoring criteria for parent and teacher VADRS. We also calculated the number of symptom criteria (items scored at 2 or 3) for ADHD and ODD scales as well as the numerical totals for ADHD and ODD symptoms scales, reporting separate counts for both parent and teacher informants. Then, we computed cross-tabulations of psychiatric diagnoses of ADHD and ODD, referencing CAPs' diagnoses against ADHD and ODD diagnoses derived from the parent and teacher VADRS scores. Cohen's Kappa was calculated to evaluate the degree of concordance between the VADRS and CAP diagnoses. To understand diagnostic discrepancies between CAP and VADRS-derived diagnoses, we further examined ADHD and ODD total scores as well as the numbers of impairment domains from parent and teacher VADRS, comparing groups of patients with/without CAP diagnosis of ODD, using ANOVAs and *t*-tests to evaluate these subgroup differences in VADRS scores. Finally, to evaluate whether CAPs' treatment practices are consistent with recognized guidelines for management of ADHD and ODD, we compared their recommendations for medication and/or behavior therapy as a function of the presence or absence of ODD, examining these recommendations against CAP-rendered ODD diagnoses *vs* VADRS-rendered ODD diagnoses.

RESULTS

ADHD diagnosis and the VADRS

As hypothesized, an initial comparison of parent and teacher VADRS scores indicates that a majority of CAP-diagnosed subjects (92%) met full ADHD diagnostic criteria on either the parent or teacher VADRS. Nearly half of the children (44%) met ADHD criteria on both the parent and teacher VADRSs (Table 1). Five children did not meet full DSM criteria regardless of the informant. A single chi-square for the overall table was χ^2 = 0.6, df = 1, P = 0.5. The observed percentage agreement between parent and teacher VADRSs was 52.4%, slightly less than expected percentage agreement by chance alone (56.4%). Calculated agreement by Cohen's Kappa was -0.09, indicating "disagreement" between parents and teachers (95%CI: -0.33 to 0.14).

Of note, DSM requires that a child meets full diagnostic criteria, with sufficient symptoms and impairment in at least one setting, and that the child also presents several ADHD symptoms in at least one other setting. Thus, for 28 of 63 subjects where both parent and teacher rating scales were positive, the child psychiatrists' diagnoses were strongly supported, with evidence of concurrent validity with strong evidence of functional impairments in both parent and teacher impairment domains (Table 2)[5]. Of note, there were 30 cases in Table 1 with only one of the two informants'

Table 2 Total attention deficit hyperactivity disorder scores, parent and teacher Vanderbilt attention deficit hyperactivity disorder diagnostic rating scale by positive *vs* negative diagnostic results (*n* = 63)

Vanderbilt rating scale	ADHD	*n*	Parent VADRS total ADHD score	Teacher VADRS total ADHD score	No. of parent's impairment domains	No. of teacher's impairment domains
Parent	Negative	5	16.6	10.2	3.4	1.8
Teacher	Negative				(SD ± 0.9)	(SD ± 1.5)
Parent	Negative	13	26.4	32.6	1.1	4.4
Teacher	Positive				(SD ± 0.5)	(SD ± 1.9)
Parent	Positive	17	33.7	16.6	2.9	1.7
Teacher	Negative				(SD ± 0.4)	(SD ± 1.6)
Parent	Positive	28	37.3	34.0	3.7	5.7
Teacher	Positive				(SD ± 1.8)	(SD ± 1.6)
Statistics			11.3	21.4	7.5	24.7
F ratio, df = 3			($P < 0.0001^1$)	($P < 0.0001^1$)	($P < 0.0003^1$)	($P < 0.0001^1$)
Total mean score (%)			32.4	27.1	2.9	4.0

[1]Statistically significant at a significance level of 0.05. ADHD: Attention deficit hyperactivity disorder; VADRS: Vanderbilt ADHD diagnostic rating scale.

Table 3 Comparison of psychiatrists' diagnoses of oppositional defiant disorder with parent or teacher Vanderbilt attention deficit hyperactivity disorder Diagnostic Rating Scale *n* (%)

		Psychiatrist ODD diagnosis		Total
		Negative	Positive	
Vanderbilt ODD screening	Negative	27 (46)	3 (5)	30 (51)
	Positive	17 (29)	12 (20)	29 (49)
Total		44 (75)	15 (25)	59

ODD: Oppositional defiant disorder.

ratings met full diagnostic symptom criteria (17 parent positive/teacher negative; 13 parent negative/teacher positive).

To better understand whether these cases showed sufficient evidence of a presence of several ADHD symptoms in at least two settings, we computed the parent and teacher total ADHD scores (adding all 18 ADHD items) for all groups as a function of positive/negative VADRS scores. The results in Table 2 indicate that even when one informant shows "negative results", that informant's total scores are still highly elevated, generally just below diagnostic threshold, providing supportive evidence of concurrent validity of CAPs' ADHD diagnoses for these 30 cases.

ODD diagnosis and the VADRS

Among 69 ADHD patients, 59 subjects had completed ADHD and ODD rating scales. Twenty-nine children scored positive for ODD on the VADRS, but CAPs confirmed the ODD diagnosis in only 12 (less than half) of these case-positives, as well as in three additional ODD-negative children. As seen in Table 3, the actual number of observed agreements between CAP and VADRS was 39 (66%), a slight improvement in the number of agreements expected by chance. The calculated agreement by Cohen's Kappa was 0.32, which is considered "fair" (95%CI: 0.10-0.53). A single χ^2 of the over all table was $\chi^2 = 7.7$ ($P = 0.0057$).

To better understand the discrepancies seen in the VADRSs' and CAPs' ODD diagnoses (Table 3), we calculated the total ODD scores from the parents and teachers' VADRS, comparing their mean total scores across the 4 ODD diagnostic agreement/disagreement categories. Four parents' total ODD scores and two teachers' total ODD sores were missing for incomplete scoring. As seen in Table 4, when comparing parent and teacher total ODD scores, parents rated children with more ODD symptoms than teachers across all of the agreement/disagreement categories. As expected, when both the CAP and the VADRS scales were in agreement for a positive ODD diagnosis (12 cases, fourth row), total parents and teachers' VADRS scores were elevated. However, surprisingly, another 17 cases had similarly elevated parent ODD scores and evident functional impairments on the VADRS, but they were not diagnosed by CAPs with ODD, a particularly surprising finding in view of the fact that CAPs did diagnose 5 additional subjects with ODD, even though their average parents and teachers' ODD scores and number of impairment domains were generally lower than the aforementioned 17 subjects.

Treatment for ADHD Children with ODD symptoms

As seen in Table 5, medication therapy was recommended to almost all (54/59) patients regardless of their ADHD/ODD diagnostic groupings. For 27 patients who did not have ODD, neither by VADRS nor CAP diagnosis, a slight majority (15) received recommendations for medication treatment alone, with most of the remainder receiving recommendations for combined treatment (medication and behavior therapy). In contrast, for the three other diagnostic groups with either a positive VADRS for ODD, a CAP diagnosis of ODD or both, the major treatment recommendation made by CAPs was for combined treatment. A single chi-square for the overall table was Pearson $\chi^2 = 22.8$, df = 6, $P = 0.0009$.

Table 4 Total parent and teacher oppositional defiant disorder scale scores, grouped by positive/negative oppositional defiant disorder diagnostic criteria, child and adolescent psychiatrist *vs* vanderbilt attention deficit hyperactivity disorder diagnostic rating scale

Diagnostic source	ODD	*n*	Parent VADRS total ODD score	Teacher VADRS total ODD score	No. of parent's impairment domains	No. of teacher's impairment domains
CAP VADRS	Negative Negative	27	6.1	1.1	2.3	3.7
CAP VADRS	Negative Positive	17	14.2	4.0	3.7	3.9
CAP VADRS	Positive Negative	3	4.0	2.0	3.3	2.3
CAP VADRS	Positive Positive	12	15.2	10.3	3.2	5.4
Statistics *F* Ratio, df = 3			24.9 ($P < 0.0001$[1])	12.0 ($P < 0.0001$[1])	2.4 ($P = 0.07$)	1.9 ($P = 0.14$)
Total mean score (%)			10.2 (10.2/24 = 43%)	3.8 (3.8/30 = 13%)	2.9	4.0

[1]Statistically significant at a significance level of 0.05. ODD: Oppositional defiant disorder; CAP: Child and adolescent psychiatrist; VADRS: Vanderbilt ADHD diagnostic rating scales.

Table 5 Psychiatric treatment recommendations for attention deficit hyperactivity disorder patients with *vs* without oppositional defiant disorder comorbidity (*n* = 59)

Diagnostic source	ODD	*n*	Medication therapy only	Behavior therapy only	Combined treatment
CAP VADRS	Negative Negative	27	15 (25%)	1 (2%)	11 (19%)
CAP VADRS	Negative Positive	17	3 (5%)	0 (0%)	14 (24%)
CAP VADRS	Positive Negative	3	0 (0%)	1 (2%)	2 (3%)
CAP VADRS	Positive Positive	12	0 (0%)	3 (5%)	9 (15%)
Total (%)			18 (30%)	5 (9%)	36 (61%)

ODD: Oppositional defiant disorder; CAP: Child and adolescent psychiatrist; VADRS: Vanderbilt ADHD Diagnostic Rating Scales.

To examine this relationship, we did a posthoc analysis using a 2 × 2 contingency table to examine the frequency of behavioral therapy recommendations (either alone or with combined treatment) as a function of the presence/absence of an ODD diagnosis; thus, 29 of 32 (91%) subjects with a possible ODD diagnosis received a treatment recommendation that included behavior therapy *vs* only 12 of 27 (44%) subjects without a possible ODD diagnosis (Pearson's $\chi^2 = 14.7$, $P = 0.0001$).

DISCUSSION

Our study examined the CAPs diagnostic practices and treatment recommendations for ADHD and its most common comorbidity, ODD, comparing their clinical diagnoses with the results of parent- and teacher-completed VADRS. We also examined CAPs' treatment recommendations against current ADHD treatment guidelines. To our best knowledge, this is the first study to examine CAPs' decision-making processes in a real-world, clinical practice setting where highest quality, specialized care for ADHD might be expected.

ADHD diagnostic findings

As anticipated, in this sample of children presenting to the ADHD clinic and diagnosed with ADHD by child psychiatrists, 91% of subjects had completed parent and teacher VADRSs available for scoring at the initial visit. Among them, the vast majority of subjects (92%, 58 of 63) met full ADHD diagnostic criteria in at least one setting (parent or teacher) on the rating scales (Table 1). Nearly half of the patients even met full ADHD diagnostic criteria in more than one setting (*e.g.*, home and school). Of note, in the case of ADHD, five conditions must be met before a diagnosis should be made: (1) The child must manifest sufficient symptoms of inattention and/or hyperactivity-impulsivity; (2) several ADHD symptoms must be present in at least two settings (*e.g.*, home, school, with peers, *etc.*); (3) the symptoms must be of sufficient duration (> 6 mo); (4) symptoms must begin during childhood (DSM-IV - before age 7, DSM-5 - before age 12); and (5) other likely or possible explanations for the symptoms must be ruled out during the evaluation (*e.g.*, vision or hearing problems, child abuse, family chaos, *etc.*). Although the DSM diagnostic criteria require that a child meets the symptom criteria threshold (*e.g.*, at least six of nine symptoms), they do not specify that the minimum six be met in more than one setting. Instead, the criteria require the presence of several ADHD symptoms in more than one setting. This two-setting requirement

serves to eliminate "setting-specific" ADHD, which may be associated more with environmental factors rather than intrinsic factors.

To examine whether the 30 children evincing full ADHD symptoms in only one setting had some ADHD symptoms in one other setting, we examined the parents and teachers' total ADHD scores, comparing those children with potentially "setting specific" ADHD to those meeting full criteria in both home and school settings, and finding comparably high symptom levels in all groups (Table 2). These findings provide supportive evidence for the concurrent validity of CAPs' ADHD diagnoses. Furthermore, the fact that similar numbers of patients were identified as meeting full criteria by either parents (n = 17, 27%) or teachers (n = 13, 21%), may reflect equivalent referral pressures from both sources, and does not support uninformed claims as seen in the media that ADHD is due to single causes, such as bad parents or unskilled teachers. Furthermore, among these 30 subjects, parents or teachers reported ADHD symptom scores were associated with the scores of performance domains reported by the same informant. In ADHD subjects with both screenings positive on the parents and teachers' VADRSs, their functions were severely impaired across the settings. Indeed, previous studies showed that ADHD symptoms were associated with executive function (EF) impairments, which are closely related to performance levels in daily activities in both home and school settings[26]. Yet, we should not ignore the variable behaviors reported by different sources in different settings, which are most likely due to interactions of intrinsic factors and environmental factors such as parent-child relationship qualities or expectation levels of parents or teachers. The considerations of these environmental factors could be more important when providers organize individualized treatment plans. Karpenko *et al*[27] concluded that without having significant treatment response to ADHD symptoms, some of the functional domains still improved reliably. This study suggests that treatment goals should focus not only on ADHD symptoms but also on better functioning in different settings[27].

Only 8% of the patients (n = 5) had both negative parent and teacher ADHD VADRS scores. Among these five cases, an in-depth review indicated that most of these patients were classified as ADHD, inattentive subtype, and tended to be comorbid with learning disorders or other chronic diseases. The parents of these five subjects reported relatively high impairments in their children's functions at home.

ODD diagnostic findings

Our findings related to CAPs' ODD diagnoses raise interesting questions: In this ADHD sample, CAPs diagnosed ODD in one-fourth of cases, substantially less than the expected 54% to 84% prevalence rates of ODD found within ADHD patients reported in other clinical sample studies[4]. In contrast to CAPs' diagnoses, nearly half of our subjects had a positive ODD screening result in either the parent or teacher VADRS, consistent with the possible conclusion that CAPs under-diagnosed ODD in the patients referred to the clinic.

Further evaluation of the 17 cases with a positive VADRS ODD rating scale but not diagnosed with ODD by CAPs revealed that these cases had much higher ODD rating scale scores on the parent VADRS than on the teacher VADRS (Table 4). Although the teacher VADRS ODD scores were lower in these cases, they were nonetheless quite comparable to the average teacher scores reported in three cases where CAPs did make the diagnosis of ODD despite negative ODD screening results. Additionally, the impairment domains reported by parents and teachers were similarly high in all groups, which is an interesting finding considering the association of ADHD score results and performance levels.

In reviewing these findings, we considered the possibility that if only the parents experience disruptive symptoms in their children's behaviors, CAPs might consider the disruptive symptoms to reflect problems in family functioning and parenting skills *vs* a disorder more intrinsic to the child. Another possible consideration that might lead to ODD "under-diagnosis" could be that CAPs do not follow ODD diagnostic criteria per se and are reluctant to make the diagnosis if teachers do not also highly rate ODD symptoms. The fact that most of the CAP-diagnosed, ODD-positive cases had both high parent and high teacher ODD symptom scores (Table 4) is consistent with these interpretations.

To further understand these findings, we considered the possibility that CAPs might tend to dismiss the possibility of an ODD diagnosis if children's ODD symptoms were noted only (or principally) by parents rather than by teachers. To examine this possibility, we conducted a posthoc analysis to assess the likelihood of CAPs making an ODD diagnosis with *vs* without a positive teacher VADRS ODD score. Accordingly, these analyses indicated that CAPs made the ODD diagnosis in 57% (4 of 7) cases where the teacher VADRS ODD score was positive, in 36% (8 of 22) cases with only a positive parent VADRS ODD score, and in 10% (3 of 30) cases with neither parent nor teacher positive VADRS ODD scores (Pearson χ^2 = 8.9, df = 2, P = 0.01).

These results suggest that negative ODD screening results from the parent and teacher VADRS has high specificity (27/30 = 90%) in ruling out ODD among patients with ADHD, consistent with earlier reports[13]. However, in this study, CAPs do not appear to rely on parent VADRS ODD screening results to rule ODD in, but do appear to place significant weight on teachers' ODD screening results - a possible decisional component that is NOT found in the DSM criteria. Moreover, the generally low prevalence rate of ODD, as diagnosed by CAPs in this study, raises questions for future research about how, when, and why CAPs make the diagnosis of ODD.

Treatment recommendation findings

In regard to treatment recommendations, for 27 children with no ODD diagnosis made by either CAP or VADRS, more than half of them were recommended for medication only, which can be seen as a conservative approach for an academic psychiatry center. In a previous study examining community-based pediatric care for ADHD, only 17% of children received behavior therapy[28]. In contrast, where CAPs made the diagnosis of ODD, or where the parent or teacher VADRS was positive for ODD, almost all of the patients received recommendations for combined therapy (medication plus behavior therapy), except for a few cases. In four cases, CAPs recommended behavior therapy only without medication for children with ODD symptoms - a group of patients consisting of younger children (mean age of 6.4 years, range 4 to 8 years).

In summary, our findings suggest that CAPs appear to follow diagnostic criteria for ADHD but not for ODD, given our results showing a relatively low prevalence rate of ODD diagnosis for ADHD, and discrepancies between CAPs' ODD diagnoses, with positive ODD screening results on the VADRS. Despite CAPs' apparent failure to make ODD diagnoses, for most of these children with ODD symptoms, whether detected by the CAPs or VADRS, CAPs nonetheless recommended behavior therapy. However, their failing to make ODD diagnoses when appropriate could lead others to underemphasize or even overlook the importance of the role of behavior therapy, *e.g*., neglecting the education of parents in understanding disruptive behaviors and learning necessary parenting skills to manage such behaviors.

Our findings suggest that the rating scales are important in real-world clinical practices as efficient and reliable means of obtaining valid information from both parents and teachers to assist CAPs in making better diagnoses of ADHD and its most common comorbidity, ODD.

We note several limitations intrinsic to this study. First, this was a retrospective study in which non-ADHD patients were excluded. Although we acquired highly completed parent and teacher VADRSs, collected at the initial evaluation in the ADHD clinic, and conducted rigorous medical-record evaluations to assess the accuracy of diagnoses in the ADHD patients, for all that, our results can only explain the accuracy of the diagnoses in the ADHD samples but not the reasons for which ADHD was not diagnosed in the non-ADHD samples. Second, our sample size was relatively small, and subjects were drawn from a single clinic, which may limit the generalizability of our findings. However, the fact that this study was conducted in an academic center psychiatric clinic, where practice standards presumably should be fairly uniform and high, suggests that future studies are needed across a broader range of clinics and clinical settings. Thus, additional research should further describe and evaluate CAP's diagnoses and treatments for ADHD within larger samples and a wider range of community-based settings. Moreover, future studies should address these same issues within the outpatient practices of primary care providers, where even greater discrepancies might be noted.

CAPs' ADHD diagnoses have strong concurrent validity against valid rating scales, but ADHD's most common comorbid condition - ODD - may be under -recognized.

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COMMENTS

Background

The American Academy of Child and Adolescent Psychiatry and AAP guidelines for attention deficit hyperactivity disorders (ADHD) practices have emphasized the importance of accurately diagnosing ADHD and its comorbidities to provide appropriate treatment options for ADHD children. However, its most common comorbidity, oppositional defiant disorder (ODD), has been reported with a wide range of prevalence rates due to a heterogeneity of the research outcome measures of ODD symptoms. Because ADHD children comorbid with ODD are at greater risk for social and academic dysfunctions, diagnosing ODD within ADHD early on is critical to prevent the externalizing behaviors from progressing. Furthermore, implementations of the established diagnostic and treatment guidelines for ADHD practices have been examined in primary care settings but not in a setting of real-world child and adolescent psychiatric practice.

Research frontiers

To make the time-consuming diagnosing process more efficient and accurate, the parent- and teacher-reported Vanderbilt ADHD diagnostic rating scales, (VADRSs) known to have high concurrent validity with ADHD diagnoses (γ = 0.79), were introduced to ADHD clinical practices. It has been suggested that the current comorbidity-scoring system in the VADRSs is excellent at ruling out ODD/CD but not at ruling in ODD/CD. However, Becker *et al*[13] examined the utility of the VADRSs to assess ODD/CD in relation to ADHD and found that a total score > 10 of the 8 ODD items on the parent VADRS, which was not in the original scoring instruction, showed high sensitivity and specificity against C-DISC-IV diagnoses for ODD.

Innovations and breakthroughs

Given the previous studies' results of high agreement of specific VADRSs thresholds against DISC-derived diagnoses, The authors compared the results of the parent and/or teacher VADRSs with clinicians' final diagnoses of these conditions. The authors also reasoned the physicians' treatment recommendations by comparing them with established treatment guidelines for ADHD and ODD practices. Theirs findings suggest that child and adolescent psychiatrists (CAPs) follow the guidelines for ADHD diagnoses and treatments but not entirely for ODD.

Applications

The VADRSs are an efficient tool to assist ADHD practices in diagnosing ADHD and ODD. CAPs and primary care physicians can gain more benefits through understanding the strengths and weaknesses of the VADRSs. Clinicians'

attentions to the ODD scorings will help to identify more ODD cases within ADHD. Further research to examine the best practice of ODD in ADHD children is required.

Terminology

ADHD: ADHD is a condition which has difficulties with attention, increased activity, and impulsivity; Cohen's Kappa: Cohen's Kappa is a statistic measure of the agreement between two groups who rate categorical data as agreed or disagreed, considering the agreement by chance alone; Concurrent validity: Concurrent validity is a measure of the extent to which a particular test correlates with a previously established measure; Confidence interval (CI): CI is a type of interval estimate of a population parameter; ODD: ODD is a condition which includes an ongoing pattern of defiant behavior toward authority figures that disturb the children's daily functioning.

Peer-review

Very nice written paper with high clinical impact.

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ORIGINAL ARTICLE

Retrospective Study

Poor CD4 count is a predictor of untreated depression in human immunodeficiency virus-positive African-Americans

Sasraku Amanor-Boadu, MariaMananita S Hipolito, Narayan Rai, Charlee K McLean, Kyla Flanagan, Flora T Hamilton, Valerie Oji, Sharon F Lambert, Huynh Nhu Le, Suad Kapetanovic, Evaristus A Nwulia

Sasraku Amanor-Boadu, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD 21205, United States

MariaMananita S Hipolito, Narayan Rai, Charlee K McLean, Evaristus A Nwulia, Department of Psychiatry and Behavioral Sciences, Translational Neuroscience Laboratory, Howard University, Washington, DC 20060, United States

Kyla Flanagan, Flora T Hamilton, Family and Medical Counseling Service, Inc., Washington, DC 20020, United States

Valerie Oji, Lifefountain Center Ministries, Inc., Katy, TX 77450, United States

Sharon F Lambert, Huynh-Nhu Le, Department of Psychology, George Washington University, Washington, DC 20052, United States

Suad Kapetanovic, National Institutes of Health, National Institute of Mental Health, Bethesda, MD 20892, United States

Author contributions: Amanor-Boadu S and Nwulia EA contributed equally to this work; Hipolito MS, Rai N and McLean CK collected and analyzed the data, and drafted the manuscript; Kapetanovic S and Nwulia EA provided analytical oversight; Nwulia EA designed and supervised the study; Amanor-Boadu S, Kapetanovic S and Nwulia EA revised the manuscript for important intellectual content; Rai N, McLean CK and Flanagan K offered the technical or material support; Hipolito MS, Rai N and McLean CK provided administrative support; all authors have read and approved the final version to be published.

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Correspondence to: Evaristus A Nwulia, MD, MHS, Associate Professor of Psychiatry Howard University, Department of Psychiatry and Behavioral Sciences, Translational Neuroscience Laboratory, Howard University, 2041 Georgia Ave., NW, 5th Floor, Washington, DC 20060, United States. enwulia@howard.edu
Telephone: +1-202-8651742
Fax: +1-202-8653068

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Abstract

AIM: To determine if efforts to improve antiretroviral

therapy (ART) adherence minimizes the negative impact of depression on human immunodeficiency virus (HIV) outcomes.

METHODS: A cross-sectional study of a clinic-based cohort of 158 HIV seropositive (HIV+) African Americans screened for major depressive disorder (MDD) in 2012. CD4 T lymphocyte (CD4+) counts were obtained from these individuals. Self-report on adherence to ART was determined from questionnaire administered during clinic visits. The primary outcome measure was conditional odds of having a poorer CD4+ count (< 350 cells/mm^3). Association between CD4+ count and antidepressant-treated or untreated MDD subjects was examined controlling for self-reported adherence and other potential confounders.

RESULTS: Out of 147 individuals with available CD4+ T lymphocyte data, 31% hadCD4+ count < 350 cells/mm^3 and 28% reported poor ART adherence. As expected the group with > 350 cells/mm^3 CD4+ T lymphocyte endorsed significantly greater ART adherence compared to the group with < 350 cells/mm^3 CD4+ T lymphocyte count (P < 0.004). Prevalence of MDD was 39.5% and 66% of individuals with MDD took antidepressants. Poor CD4+ T lymphocyte count was associated with poor ART adherence and MDD. Adjusting for ART adherence, age, sex and education, which were potential confounders, the association between MDD and poor CD4+ T lymphocyte remained significant only in the untreated MDD group.

CONCLUSION: Therefore, CD4+ count could be a clinical marker of untreated depression in HIV+. Also, mental health care may be relevant to primary care of HIV+ patients.

Key words: Human immunodeficiency virus positive; Depression; CD4 T lymphocyte count; Antiretroviral Therapy; African Americans

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Core tip: A retrospective data review was done on human immunodeficiency virus+ patients of a primary care clinic. We examined data on depression diagnosis of patients over a two-year period. Antiretroviral therapy (ART) adherence and major depressive disorder were associated with CD4+ lymphocyte counts. Non-treatment of depression was associated with poor CD4+ lymphocyte count independent of ART adherence.

Amanor-Boadu S, Hipolito MS, Rai N, McLean CK, Flanagan K, Hamilton FT, Oji V, Lambert SF, Le HN, Kapetanovic S, Nwulia EA. Poor CD4 count is a predictor of untreated depression in human immunodeficiency virus-positive African-Americans. *World J Psychiatr* 2016; 6(1): 128-135 Available from: URL: http://www.wjgnet.com/2220-3206/full/v6/i1/128.htm DOI: http://dx.doi.org/10.5498/wjp.v6.i1.128

INTRODUCTION

Human immunodeficiency virus (HIV) epidemic is a major public health concern, with significant clinical, social and economic impacts, disproportionately affecting minority populations who have less access to care. Approximately 1.2 million people are living with HIV in the United States[1] with 50000 new infections yearly and 1 out of 5 infected people unaware of their status[2]. Since its discovery, HIV/AIDS (acquired immunodeficiency syndrome) has been studied and researched widely and there have been significant improvements in our knowledge of HIV disease and its treatment. The advent of highly active antiretroviral therapy (HAART) has made it possible to suppress the infection, thus prolonging life and putting HIV in the class of chronic long-term infections for many individuals.

Evidence strongly suggests significant association between HIV infection and depression[3]. Prevalence rates of depression are significantly higher in HIV-infected (HIV+) individuals than in the general population[4-7]. The prevalence of depressive symptoms or mood disorders in HIV+ individuals may be about 30% in the United States[4,8]. These prevalence rates vary across specific population groups in HIV+ individuals, with studies reporting higher prevalence rates in HIV+ women than in HIV+ men[3,9,10] and increased depression risk among HIV+ individuals with substance abuse disorders[4,11,12] and among those who have same-sex sexual partners[13,14]. While the prevalence rate of depression decreases with increasing age for the general adult population, this may not be the case for HIV+ adults as evidenced in some studies[15]. Although earlier studies failed to depict this relationship, recent longitudinal studies, especially since the advent of HAART, have observed significant associations between depression and markers of poorer HIV disease outcomes including lower CD4 counts and higher viral loads[16]. The reported associations between depression and HIV disease severity might be mediated by risky behaviors, such as substance abuse, unsafe sexual practices or ART non-adherence, which may further increase transmission risk or exacerbate infection in HIV+ individuals - especially with new drug-resistant viral strains. Indeed, majority of current studies on the subject suggest that co-morbid depression may negatively impact adherence to ART, while non-adherence to HAART, depression and lack of antidepressant use have been associated with accelerated progression of HIV[16-18] and increased mortality[19-21]. On the other hand, given the growing body of scientific evidence implicating important role of immunologic mechanisms in the pathophysiology of major depressive disorder (MDD)[22-24], especially in people with chronic illnesses, it is plausible that the association between MDD and CD4+ outcome could be driven by immunologic mechanisms that are independent from ART adherence and other relevant health behaviors.

This study was conducted with a clinical cohort

of African-American HIV+ individuals with or without MDD comorbidity, who reside in a community that has received intense focus and concerted efforts to improve HIV screening, treatment and ART adherence. In an effort to determine if integrating specialized depression management is needed in addition to intense ART adherence promotion, we collected clinical and immunologic data to examine the indirect and direct associations between MDD and CD4+ counts, adjusting for ART adherence. We hypothesized that non-adherence to ART and non-treatment of co-morbid depression would be independently associated with poor CD4+ levels on admission.

MATERIALS AND METHODS

Study site and sample

The study sample consisted of adult HIV+ African Americans who received care at the Family and Medical Counseling Service (FMCS), Inc. during years 2011 and 2012, and who were formally screened for depression during their intake registration. The FMCS, Inc. is a primary care center that provides health services for the largely African American communities living in the South-East zone district of Washington, DC. A blood specimen was collected for each patient at entry, for measurement of viral load, immunologic profiles and other routine tests. All participants completed a detailed biopsychosocial form, comprised of sociodemographic, behavioral, and medical information. All collected data were stored in an encrypted and password-protected electronic medical record (EMR). To search for additional information missing in the EMR, such as names of ART prescribed prior to intake admission and names of abused substances, we reviewed patient paper documents and charts with handwritten notes. This retrospective study was approved by the Howard University Institutional Review Board.

Assessment of depression

As part of their intake registration into the clinic between 2011 and 2012, patients were interviewed with the Substance Abuse and Mental Illness Symptoms Screener (SAMISS), a well-validated 16-item clinician-administered questionnaire that screens for patterns of substance abuse and key psychiatric syndromes, including manic and major depressive episodes, generalized anxiety disorder, panic disorder, post-traumatic stress disorder and adjustment disorder[25]. Additionally, the SAMISS inquires about use of antidepressants in the past year. It is usually administered in 10 min, and has exhibited good psychometric properties[26]. All intake clinical personnel at FMCS were trained to administer the SAMISS in a reliable manner. Patients interviewed with the SAMISS were asked if they had experienced a period lasting two weeks or more in the past year when they felt depressed, and then subsequently asked if they received a diagnosis or treatment(s) for depression. An affirmative response to a two-week period of depression was used for classification of MDD in this study. All patients who screened positive for MDD in the past year either received antidepressants or a physician's diagnosis of MDD, thereby providing some support to the validity of self-report of MDD in this questionnaire.

Other assessments

CD4+ T lymphocyte counts were measured from blood specimen collected from each patient on the day of clinic intake and during follow-up visits. These results were abstracted from the EMR, were all clinical and laboratory data were stored. We used the most recent CD4+ count for the purpose of this study.

ART adherence and other potential correlates of CD4+ outcome, including age, gender, education, monthly income, housing, and insurance status were derived from the biopsychosocial section of the EMR. As part of their intake interview and during subsequent primary care visits, all patients regardless of their HIV status were asked if they knew about their HIV status, and if they were receiving HIV treatments. Patients who acknowledged being HIV positive were subsequently asked what percentage of time they took their HIV treatments as prescribed by their physicians. For the purpose of this study, we used 80% adherence as threshold for classification of adequacy of ART adherence: $\geqslant$ 80% adherence was classified as good or adequate adherence and < 80% was classified as poor or inadequate adherence. Adherence status for subjects who recently became seropositive for HIV was classified as unknown. Substance abuse and pattern of alcohol use were determined from responses to questions in the SAMISS. Problem drinking was defined as heavy drinking (*i.e.*, > 4 drinks per drinking episode) in addition to having experienced difficulty cutting down on drinking or social problems as a consequence of drinking. Additionally, two questions in the SAMISS specifically assessed abuse of prescription and nonprescription drugs in the past year. Presence or absence of substance abuse history was determined from questions in SAMISS inquiring about problem drinking, illicit drug use, and abuse of prescription drugs.

Statistical analysis

The STATA statistical package, version 12 was used to carry out this analysis. The CD4 count data was incomplete with 11 missing values. Since our outcome of interest is CD4 count differences across groups, we excluded subjects with missing information on CD4 count from the regression analyses. We could efficiently do so because the individuals with missing CD4 count values had similar distribution as non-missing across other variables in the data set.

Age, monthly income, and CD4 T lymphocyte count were grouped into categories because of their highly skewed distributions. Age was thus categorized into < 35 years, 35-55 years and > 55 years. Monthly income was grouped into categories according to quartiles. CD4 count was categorized into < 350 cell/mm^3

Table 1 Demographic and behavioral characteristics of study participants with and without a CD4 count of < 350 cells/mm^3

Characteristics	CD4 count >350 (*n* = 102) %	CD4 count < 350 (*n* = 45) %	*P*
Gender			
Male	62.75	51.11	0.19
Female	37.25	48.89	
Age groups (yr)			
< 35	12.75	17.78	0.16
35-55	65.69	73.33	
> 55	21.57	8.89	
Monthly income ($)			
0-200	35.11	27.27	0.72
204-670	15.96	22.73	
672-739	25.53	25	
743-2650	23.4	25	
Educational status[1]			
College	22.22	42.31	0.049
No college	77.78	57.69	
HIV treatment adherence			
No	20.59	44.44	0.004
Yes	50	44.44	
Unknown	29.41	11.11	
Substance abuse			
No	38.24	44.44	0.48
Yes	61.76	55.56	
Problem drinking			
No	63.73	61.36	0.79
Yes	36.27	38.64	
Depression			
No	66.67	46.67	0.02
Yes (treated)	24.51	28.89	
Yes (untreated)	8.82	24.44	

[1]Data on education was missing in 49 patients. HIV: Human immunodeficiency virus.

("poor CD4+ count") and > 350 cells/mm^3 based on earlier findings in studies, that observed that deferring treatment until CD4 count was < 350 cells/mm^3 was associated with worse prognosis and mortality[27-29].

χ^2 and Fisher's exact test were used to compare social, demographic and behavioral factors by CD4+ status. For all bivariate analyses, a *P* value of < 0.05 was regarded as threshold for significance. With the exception of education status and insurance status, missing response rates were less than 8%. Educational status was missing in 33% of individuals and insurance status was missing in 55% of individuals; hence, these latter variables were not included in our multivariate analysis.

We used multiple logistic regression analysis to determine the independent association of MDD with HIV immunological status, adjusting for MDD treatment, ART adherence, age and gender. Several epidemiologic studies have implicated age and gender as key determinants of MDD and HIV outcomes; hence our decision to include these two variables in our multivariate model[30,31]. Ethnicity, another key determinant was not included because our study base consisted of 99% African Americans. The primary outcome measure of interest was the adjusted OR of poor CD4+ outcome comparing MDD treatment groups to nonMDD controls.

RESULTS

Sample characteristics

From October 2011, when depression screening with SAMISS was first implemented in the FMCS, to July 2012, a total of 158 HIV+ patients were enrolled into treatment, and all 158 (100%) of them completed the SAMISS. The 11 individuals who were missing information on CD4+ counts were excluded, resulting in the final sample size of 147. Table 1 depicts the distribution of demographic and behavioral variables by CD4+ count categories among the 147 individuals included in the analysis. As shown, a total of 45 (31%) patients had baseline CD4+ count < 350 cell/mm^3. Majority of the patients were males, between 35 and 55 years of age, and with low monthly income; no significant differences for age, sex or monthly income were observed by CD4+ categories. Group differences were, however, observed for education attainment and ART adherence. Twenty-two percent of those with CD4+ count > 350 cells/mm^3 received some college education, compared to 42% among those with CD4+ count < 350 cells/mm^3 ($P < 0.05$). However, data on education was missing in 49 patients. As depicted in Table 1, among the patients with CD4 count > 350 cells/mm^3, 21% reported poor ART adherence and 50% reported good ART adherence. The ART adherence status of the remaining 29% of patients was classified as "not applicable", since this group consists of patients with recent HIV+ diagnosis, who have not been prescribed ART. Among the category of patients with poorer CD4+ counts (*i.e.*, < 350 cells/mm^3), the proportion with poor adherence is reversed, accounting for 44%. Approximately 24% of patients in the poor CD4 group had untreated depression in the past year, compared to 9% of untreated depression among those with CD4+ count > 350 (Table 1). In addition, 53% of patients in the poor CD4 group had past year MDD diagnosis, compared to 33% past year MDD in those with CD4 count > 350 cells/mm^3.

Table 2 shows the comparison of ART adherence and other potential demographic and behavioral correlates of poor CD4 outcome between patients with treated MDD, untreated MDD and without MDD. Patients with untreated MDD had the lowest ART adherence rate. No significant differences were observed between these groups in ART adherence. ART adherence was highest among those with treated MDD. Problem drinking rates 55% for the untreated MDD, 46% for treated MDD and 28% for patients without MDD ($P < 0.02$). No significant differences were observed in age, gender, income, education and substance abuse between the MDD categories.

Relationship between depression and CD4+ counts

Table 3 depicts the result of our multiple logistic regression analyses for determination of the independent

Table 2 Demographic and behavioral characteristics of study participants with or without depression

Characteristics	No MDD (n = 89) (%)	MDD treated (n = 38) (%)	MDD untreated (n = 20) (%)	P
Gender				
Male	63.27	50	60	0.35
Female	36.73	50	40	
Age groups				
> 55	24.49	5	15	0.12
35-55	62.24	77.5	70	
< 35	13.27	17.5	15	
Monthly income				
0-200	32.61	30.77	33.33	0.32
204-670	17.39	25.64	5.56	
672-739	21.74	30.77	27.78	
743-2650	28.26	12.82	33.33	
Educational status				
No college	70.59	78.57	72.73	0.73
College	29.41	21.43	27.27	
Accommodation				
Yes	60.42	72.50	70	0.35
No	39.58	27.50	30	
HIV treatment adherence				
No	26.53	25	45	0.51
Yes	48.98	52.50	40	
Unknown	24.49	22.50	15	
Substance abuse				
No	41.84	30	45	0.37
Yes	58.16	70	55	
Problem drinking				
No	72.45	53.85	45	0.02
Yes	27.55	46.15	55	

HIV: Human immunodeficiency virus; MDD: Major depressive disorder.

association between MDD and poor CD4+ outcome, adjusting for ART adherence, age and gender. The result of simple logistic regression for each variable in the multiple regression was included to aid comparison of indirect versus direct (*i.e.*, independent) associations. In the un-adjusted analysis (second column of Table 3), the odds of having poor CD4 count in the treated MDD group were 1.68 times that of the non-MDD group, and this difference was not significant (P < 0.3). On the other hand, there was an almost 400% increase in odds of poor CD4 outcome in the untreated MDD group compared to the reference (nonMDD) group (P < 0.008). Adjusting for age, gender and ART adherence, the odds ratio (OR) of poor CD4 comparing untreated MDD to non-MDD patients was 3.12 (P < 0.04).The unadjusted analysis revealed a 60% decreased odds of poor CD4 count outcome comparing the group with good ART adherence to the group with poor ART adherence (P < 0.03). Also in the multivariate analysis, there was a significant inverse association between ART adherence and poor CD4 counts (OR = 0.38, P < 0.03). Our multiple logistic model revealed significant associations between the younger age groups (*i.e.*, < 35 years and 35 to 55 years) and poor CD4 outcome, treating the older age group (*i.e.*, > 55 years) as reference group.

Table 3 Logistic regression analysis of potential risk factors for lower CD4 count of < 350 cells/mm^3

Characteristic	OR (P-value, 95%CI)	AOR (P-value, 95%CI)[1]
Depression		
No	REF	REF
Yes (treated)	1.68 (0.22, 0.73-3.87)	1.35 (0.52, 0.54-3.38)
Yes (Untreated)	3.96 (0.008, 1.44-10.88)	3.12 (0.04, 1.07-9.11)
Age		
> 55	REF	REF
35-55	2.71 (0.09, 0.86-8.54)	3.22 (0.03, 1.14-9.11)
< 35	3.38 (0.09, 0.85-13.55)	4.25 (0.03, 1.15-15.69)
Gender		
Male	REF	REF
Female	1.61 (0.19, 0.79-3.28)	1.48 (0.33, 0.68-3.21)
HIV treatment adherence		
No	REF	REF
Yes	0.41 (0.03, 0.18-0.92)	0.38 (0.03, 0.17-0.89)
Unknown	0.18 (0.003, 0.06-0.54)	0.17 (< 0.01, 0.05-0.57)

[1]Model includes: MDD, age sex and adherence. REF: Reference category; HIV: Human immunodeficiency virus; MDD: Major depressive disorder; AOR: Adjusted odds ratio.

DISCUSSION

In this study of HIV+ individuals of African-American ethnic background, we found a 39% prevalence of positive screen for depression in the past year, higher than the prevalence rates in the general population and in keeping with previous studies. Untreated depression was associated with lower CD4 T lymphocyte counts, suggesting a significant relationship between untreated depression and immune status in HIV+ individuals. Poorer immunological status was also significantly associated with younger age and non-adherence to ART. These findings are very important as the ultimate result of lower CD4 T lymphocyte counts in HIV disease is progression to AIDS and mortality[32,33].

CD4 T lymphocytes counts are reported as the number of cells in a cubic millimeter of blood. A normal CD4 count is from 500 to 1500 cells per cubic millimeter of blood. CD4 T lymphocytes are primarily targeted by HIV viral cells and play an important role in host immune defenses against opportunistic infections[34]. With progressive HIV disease, CD4 count levels drop to low levels resulting in AIDS defining illness and increasing mortality. As such, CD4 count is one of the most important prognostic markers of HIV-1 disease progression and an important measure in the decision for commencement of ART in HIV+ patients. Our finding of reduced CD4 T lymphocyte count in association with untreated depression has potential clinical significance, as it suggests that CD4 count could potentially be used as clinical marker of untreated co-morbid depression in HIV+ individuals, in addition to its well-established value as marker of HIV disease severity[35].

Efforts to understand the role of the immune system in major depression have been going on for 3 decades. The majority of studies have focused on the

role of activated innate immune system in depression, with fewer studies on the role of T lymphocyte cells and other adaptive immune responses[36]. It is of note that some initial studies on T lymphocyte responses in hospitalized depressed patients, revealed a reduced proliferation of T cells in response to T cell mitogens - phytohemagglutinin, concanavalin A[37-39], but these findings were not always reproducible. To elucidate this discrepancy, an extensive metaanalysis of more than 180 studies comprising greater than 40 immune measures, revealed mild reductions in Natural Killer cell count, decreased T cell count and T cell proportions and reduced lymphocyte proliferation responses in depressed patients compared to their controls[40].

The evidence based data depicting T lymphocyte dysfunction in depressed individuals, furthered studies on the role of depression in HIV progression. An extensive literature review on studies carried out in both pre-HAART and post-HAART eras showed depression exacerbates HIV progression to AIDS, with at least three studies further depicting an independent association of depression with decreased CD4 T lymphocyte count in individuals with HIV[32]. An example is a longitudinal study of a multiethnic 177 HIV positive men and women, with CD4 counts of between 150 and 500, followed for 2 years. After adjusting for age, gender, antiretroviral treatment and adherence, cumulative depression and hopelessness were associated with decreases CD4 count and increase in viral load[41].

Our results also show a significant association between ART treatment adherence and CD4+ T lymphocyte count, which is in keeping with previous studies[32,42]. Individuals who were adherent were approximately 60% less likely to have a poor CD4 count in the univariate analysis and after controlling for depression, age and gender. Other studies examining different populations have found depression to be associated with poor ART adherence[41-43]. One of such studies of HIV infected injecting drug users, revealed an association between depression and ART non-adherence as a strong indicator of clinical progression[44]. Another study on indigent low income ethnic minorities with HIV, consisting of mainly African-American population, revealed a significant association between depression and poorer ART adherence[45]. Thus, some studies also discussed the possibility of ART adherence being a potential mediator of sorts in the relationship between MDD and poor CD4+ T lymphocyte outcomes[32,40,41].

In lieu of the mediating effect of ART adherence on the relationship between depression and CD4 count, our study examined if the strength of the relationship between depression and CD4 count diminished by adjusting for ART adherence. Our results confirmed an independent relationship between depression and CD4 count. There was however a 21% decrease in the odds of poorer CD4 count in the untreated depression group after adjusting for ART adherence. Hence, our study shows that ART adherence may be a confounder in the association between untreated depression and poorer CD4 count, especially in light of the fact that nearly 50% of individuals with untreated depression also were non adherent to ART. However, larger prospective studies will be beneficial in further examining the nature of these relationships.

This study has some limitations that should be acknowledged. The cross-sectional design and small sample size limit our ability to make inferences about causal direction of the observed associations and their significance, respectively. The study sample consisted exclusively of African-American individuals from a specific urban community. While this limits the generalizability of the findings, it also provides natural control for potential sociodemographic (*e.g.*, poverty, health disparities) and cultural, racial or community-bound factors that could have confounded the results. It also provides clues that may help inform future clinical and research efforts to adapt evidence based-psychological and social interventions, including mental health screening and treatment monitoring protocols in a manner that will accommodate the sociocultural dimensions of HIV, depression, and salient health behaviors that may be unique to predominantly low-income, African-American urban communities.

In summary, we found that depression is highly prevalent in this population of HIV+ individuals and untreated depression and poor ART adherence are independently associated with poorer CD4 T-lymphocyte outcomes. Furthermore, it contributes to growing appreciation of other possible pathways linking depression to HIV outcome, other than the mediating role of ART adherence. Our findings need to be validated in a larger cohort with follow up over time. There is also a need for further research into the factors that may predispose this community to poorer outcomes. The lower mean income and educational attainment in this clinical cohort also suggests the potential relevance of integrating specialized mental health and case management services into community primary care centers in for better management of HIV+ patients.

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COMMENTS

Background

Human immunodeficiency virus (HIV) epidemic is a major public health concern. Approximately 1.2 million people are living with HIV in the United States with 50000 new infections yearly. Evidence strongly suggests significant association between HIV infection and depression. Prevalence rates of depression are significantly higher in HIV-infected individuals in comparison to the general population. The reported associations between depression and HIV disease severity might be mediated by risky behaviors, such as substance abuse,

unsafe practices or antiretroviral therapy (ART) non-adherence. Consequently, these factors may further increase transmission risk or exacerbate infection in HIV+ individuals - especially with new drug-resistant viral strains. Recent studies have shown significant association between depression and markers of poorer HIV disease outcomes including lower CD4 counts and higher viral loads. In contrast, the association between major depressive disorder (MDD) and CD4+ outcome could be driven by immunologic mechanisms that are independent from ART adherence. To examine the indirect and direct associations between MDD and CD4+ count, adjusting for ART adherence, the authors collected clinical and immunologic data from the cohort of African-American HIV+ individuals with or without MDD comorbidity. The aim of this study is to see the association between non-adherence to ART and non-treatment of co-morbid depression with poor CD4+ levels on admission.

Research frontiers

Majority of the current studies suggest that co-morbid depression may negatively impact adherence to ART, while non-adherence to highly active ART, depression and lack of antidepressant use have been associated with accelerated progression of HIV and increase mortality. On the other hand, given the growing body of scientific evidence implicating important role of immunologic mechanisms in the pathophysiology of MDD, especially in people with chronic illnesses, it is plausible that the association between MDD and CD4+ outcome could be driven by immunologic mechanisms that are independent from ART adherence and other relevant health behavior.

Innovation and breakthroughs

Efforts to understand the role of the immune system in major depression have been going on for 3 decades. The majority of studies have focused on the role of activated innate immune system in depression, with fewer studies on the role of T lymphocyte cells and other adaptive immune responses. In this study, the authors found 39% prevalence for depression, which is higher than the general population. Untreated depression was associated with lower CD4 T lymphocyte counts, suggesting a significant relationship between untreated depression and immune status in HIV+ individuals. Poorer immunological status was also significantly associated with younger age and non-adherence to ART.

Applications

ART adherence may be a confounder in the association between untreated depression and poorer CD4 count. This study provides the evidence that non-adherence to ART and non-treatment of co-morbid depression would be independently associated with poor CD4+ levels on admission. Practical application: To determine if recent efforts to improve ART adherence among HIV+ African Americans attending a primary care clinic sufficiently minimizes the negative impact of depression on HIV outcomes.

Terminology

Epidemics: Widespread outbreak of infectious disease in a population or community; Cohort: Group of people with common characteristics.

Peer-review

The manuscript explores interesting relationship between CD4 count and depression. It found that there is association between poor CD4 count and untreated depression.

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ORIGINAL ARTICLE

Prospective Study

Isotretinoin was not associated with depression or anxiety: A twelve-week study

Bella Suarez, Ana Serrano, Yves Cova, Trino Baptista

Bella Suarez, Ana Serrano, Department of Psychiatry, Los Andes University Medical School, Mérida 5101, Venezuela

Yves Cova, Department of Dermatology, Los Andes University Medical School, Mérida 5101, Venezuela

Trino Baptista, Department of Physiology, Los Andes University Medical School, Mérida 5101, Venezuela

Author contributions: Suarez B and Cova Y conducted the study; Serrano A and Baptista T did the data processing and analysis; Baptista T designed the study and wrote the manuscript.

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Correspondence to: Trino Baptista, MD, PhD, Professor, Department of Physiology, Los Andes University Medical School, Tulio Febres Cordero Avenue, Merida 5101, Venezuela. trinbap@yahoo.com
Telephone: +58-274-2636395
Fax: +58-274-2639011

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Abstract

AIM: To investigate the frequency and severity of depression and/or anxiety in isotretinoin (ITT)-treated subjects and in a non-ITT control group.

METHODS: Sixty consecutively-admitted non-psychiatric outpatients with acne were assigned to either ITT at a fixed dose of 30 mg/d (n = 36) or "other treatment" group (OT; n = 24). The Zung depression or anxiety scales (with cut-off points), two locally developed scales for depression (GeDepr) and anxiety (Ansilet) (without cut-off points) and clinical global impression scales of acne severity were administered at baseline and at weeks 6 and 12 of treatment. Data was analyzed with the chi-squared test and covariance analysis.

RESULTS: Gender distribution, age, marital status and education level did not differ between both treatment groups. The frequency of depression, as defined by the Zung scale cut-off points was similar in the ITT and in the non-ITT groups: Weeks 6 and 12: 8.3% in both groups, P = 0.9. The frequency of anxiety was similar in the groups as well: Week 6: ITT = 8.3%; OT = 0.0%, P > 0.05; week 12: ITT = 11.1%, OT = 4.2%, P > 0.05. The scores in both scales' sets did not differ between the treatment groups at any evaluation time point (P

> 0.05). Five ITT-treated subjects (13.8%) and two from the OT-treated group (8.3%) developed clinically significant anxiety and/or depression during treatment (*P* > 0.05).

CONCLUSION: Our study confirms the safety of ITT regarding psychological side effects in regular dermatological patients. Susceptible subjects may exist but their identification requires additional strategies.

Key words: Isotretinoin; Short-term; Psychopathology; Depression; Anxiety; Other treatments

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Core tip: Isotretinoin (ITT) is frequently used for acne therapy, particularly in young people, but concerns exist regarding the risk of depression and suicide attempts. We conducted a 12-wk prospective study administering a fixed ITT dose in non-psychiatric acne patients an in a non-ITT control group. We used categorical and continuous scales for the assessment of depression and anxiety. The frequency and severity of psychopathology was similar in both treatment groups, stressing the safety of ITT in typical dermatological patients. However, 13.8% ITT subjects and 8.3% of the non-ITT developed clinically significant anxiety and/or depression. Hence, susceptible subjects exist, who deserve further investigation and assistance.

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INTRODUCTION

Isotretinoin (ITT) is a vitamin A-related compound widely used in the treatment of severe cystic acne vulgaris[1]. It is less frequently used to treat squamous-cell carcinoma, brain or pancreatic cancer and severe ichtyosis[2].

The safety of ITT on mental functioning has been a concern since its introduction into clinical practice. For example, as early as in 1983, clinically significant depression was reported in a case series[3].

The association between ITT treatment and psychopathology, particularly with depressive symptoms, comes from accumulated evidence of case reports, temporal association, challenge-re-challenge tests, dose response/class effect studies, and biological plausibility[4]. As a matter of fact, ITT was among the top five drugs with the most frequent spontaneous reports of depression in a recent survey conducted in the United Kingdom[5]. Collectively, these results have been criticized for not discriminating between the worsening of pre-existing psychopathology and the development of *de novo* psychopathology[4].

The frequency of depression during ITT administration observed in group studies ranges from 1% to 11%[4]. The low frequency of depression obtained in some studies has been associated with protocols based on self-reports of psychological symptoms. Research has shown that patients tend to underestimate the magnitude of depression in comparison to their family controls[6]. Besides, numerous negative studies lacked standardized diagnostic protocols and/or control groups[4].

Current research trends suggest that the risk of ITT-related depression maybe particularly relevant in a minority of patients who are also susceptible to other ITT neural side effects, such as headache[4,7]. Besides, subjects with bipolar, anxiety or obsessive-compulsive disorders may be at risk of clinical worsening during ITT administration[4,8]. By contrast, numerous practitioners and research studies report that ITT may rather improve the psychological status in patients with severe acne[4]. These results are not in contradiction with the finding of a specific minority of subjects with increased susceptibility to ITT-related psychological side effects.

In this longitudinal 12-wk study, we evaluated depression and anxiety symptoms in acne patients receiving either ITT or antibiotics by using not only the well-known Zung scales for depression/anxiety but also a set of instruments developed and previously validated at Los Andes University, Mérida, Venezuela.

MATERIALS AND METHODS

This is a naturalistic, longitudinal, open-labeled study conducted in a non-probabilistic sample from February 2013 to June 2013 at the Dermatology Department of Los Andes University Hospital in Mérida, Venezuela. It was approved by the Ethics Committee and by the Institutional Review Board of the Department of Psychiatry, Los Andes University Medical School, Mérida, Venezuela. No formal trial registration system exists in this country.

Subjects and treatments

Consecutively admitted patients consulting for acne were invited to participate in the study and filled an informed consent of voluntary participation. Treatments were assigned by a dermatologist (YC) according to disease severity and patient's income. The participating subjects were assigned either to the ITT-group (30-40 mg/d) or to the OT-group that consisted in the administration of a combination of oral or topic antibiotics and the antibacterial agent benzoil peroxide. Exclusion criteria were previous administration or intolerance to ITT, < 18 years of age, and/or explicit refusal to participate in the study.

Evaluation

Acne severity was assessed by the dermatologist at

Table 1 Demographic features

Isotretinoine (n = 36)		Other treatments (n = 24)	
Gender [n (%)][1]			
Females	Males	Females	Males
16 (44.4)	20 (55.6)	11 (45.8)	13 (54.2)
Age (mean ± SD)[2]			
23.0 ± 3.2	20.3 ± 2.9	22.9 ± 3.8	23.4 ± 4.4
Marital status [n (%)][3]			
Single	Others	Single	Others
34 (94.4)	2 (5.6)	15 (62.5)	9 (37.5)
Education [n (%)][4]			
College	Others	College	Others
6 (16.6)	35 (83.4)	4 (16.7)	(83.3)

[1]χ^2 (1) = 0.01, P = 0.9; [2]F (3) = 2.9, P = 0.04; post-hoc Tukey HSD test = 0.07; [3]χ^2 (1) = 7.8, P = 0.005; [4]χ^2 (1) = 0.00, P = 1.

baseline only by using a 5-point, likert-type, clinical global impression scale, from 0 as "minimally severe" to 5 as "very severe". At weeks 6 and 12, the patients and the treating physician assessed the changes in acne severity compared to baseline by using a 7-point, likert-type scale, from 0 as "markedly worst" to 7 as "very much improved".

Depression and anxiety were evaluated at baseline and at weeks 6 and 12 with categorical and continuous self-administered scales. The former consisted in the validated Spanish versions of the Zung depression and anxiety scales that provided a categorical classification[9-11]. Every scale consisted of 20 items with positive or negative valence that explored the frequency of depression or anxiety signs and symptoms, as follows: From 1 = rarely to 4 = always. Each subject was then classified as non-depressed or non-anxious when scoring < 50 points, or slightly, moderately or severely depressed and/or anxious when scoring 50-59, 60-69 and ⩾ 70 points, respectively.

The continuous scales corresponded to the previously mentioned Zung scales plus the following two scales for continuous quantification of depression or anxiety levels. The "Ge-Depr" is a two-factor scale consisting of 16 depression-related items. It was validated in 249 Venezuelan subjects and reported a Cronbach alpha coefficient of 0.88 and Pearson correlation coefficients of 0.65 *vs* an aggression scale, of 0.68 *vs* an anxiety scale, and of 0.65 *vs* a general scale of psychological maladjustment[12].

The "Ansilet" is a one-factor scale consisting of 15 anxiety-related items. It was validated in 264 Venezuelan university students and reported a Cronbach alpha coefficient of 0.82 and a Pearson correlation coefficient of 0.59 *vs* the self-esteem Rosemberg scale[13].

Both local scales were scored with a 6-point likert-type scale from 0 = complete disagreement to 6 = complete agreement and no neutral score. These scales did not include a cut-off point but are aimed to assess changes over time in a continuous scale.

A separate analysis was conducted with the Zung depression scale items that explore suicide-related ideation (items 17 and 19).

Statistical analysis

Categorical data (frequency of depression or anxiety according to the Zung scales from baseline to week 12 with intra-group and inter-group comparisons) were analyzed with the χ^2 test.

Continuous data (depression and anxiety scores) were analyzed with the univariate general linear model with treatment and time as between- and within-group variables, respectively, and age as covariate.

Bivariate correlation analysis was conducted with the Pearson correlation coefficient. Results were considered significant when P < 0.05.

RESULTS

Sixty consecutively admitted patients were assigned either to the ITT-group (n = 36) or to the OT-group (n = 24). No subject was excluded in any group. Gender distribution and education level were similar in both groups, but the ITT-treated males tended to be younger, and the proportion of single (unmarried) subjects was significantly higher in the ITT group (Table 1). Most patients (35 out of 36) received 30 mg/d of ITT; hence, the treatment protocol consisted of a fixed-dose schedule.

Acne severity was higher in the ITT - than in the control group. Severe and very severe acne was only observed in the ITT group: 9 subjects (25%) *vs* 0 subjects (0%): Mann Whitney test: Z = 2.6, P = 0.008.

Depression and anxiety frequency and scores

The frequency of depression or anxiety categorically defined according to the Zung scales and the scores obtained in the continuous scale analysis were similar in both treatment groups at baseline and did not display significant changes over time (Tables 2 and 3). The scores in the depression Zung scale suicide related items were similar in both treatment groups at baseline and at weeks 6 and 12: Item 17: F (1) = 0.001, P = 0.9; item 19: F (1) = 0.5, P = 0.5 (data not shown).

The Zung depression and anxiety scale scores showed a highly significant positive correlation with the GeDepr and Ansilet scale scores, respectively: The r coefficient value was over 0.60, P = 0.000 in all the analyses (data not shown).

Correlation analysis

Clinical assessment of acne severity and depression/anxiety scores: For the sake of concision, we only present the data related to the GeDepr and Ansilet scale scores.

No significant correlations were observed between the clinical severity assessment of acne at baseline and the depression and anxiety scores at any time-point in the ITT group. However, in the OT group, significant correlations were observed in the depression scores,

Table 2 Frequency of depression and anxiety according to the Zung scales at baseline and during treatment

	ITT			OT		
	Categorical level of depression [*n* (%)][1]					
	Basal	Week 6	Week 12	Basal	Week 6	Week 12
Mild	1 (2.8)	3 (8.3)	3 (8.3)	2 (8.3)	2 (8.3)	2 (8.3)
Moderate	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Severe	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Total	1 (2.8)	3 (8.3)	3 (8.3)	2 (8.3)	2 (8.3)	2 (8.3)
	Categorical level of anxiety [*n* (%)][2]					
	Basal	Week 6	Week 12	Basal	Week 6	Week 12
Mild	1 (2.8)	2 (5.5)	1 (2.8)	1 (4.2)	0 (0.0)	0 (0.0)
Moderate	0 (0.0)	1 (2.8)	2 (5.5)	0 (0.0)	0 (0.0)	0 (0.0)
Severe	0 (0.0)	0 (0.0)	1 (2.8)	0 (0.0)	0 (0.0)	1 (4.2)
Total	1 (2.8)	3 (8.3)	4 (11.1)	1 (4.2)	0 (0.0)	1 (4.2)

Values represent number and percentage in parentheses of subjects with depression or anxiety. [1]Within-group comparisons: ITT group: χ^2 (2) = 1.2, P = 0.5; OT group: χ^2 (2) = 0.0, P = 1; between-group comparison: χ^2 (5) = 1.3, P = 0.9; [2]Within-group comparisons: ITT group: χ^2 (2) = 4.6, P = 0.6; OT group: χ^2 (2) = 4.0, P = 0.4; between-group comparison: χ^2 (5) = 4.8, P = 0.4. OT: Other treatments; ITT: Isotretinoine.

and marginally significant correlations were detected in the anxiety value sets (Table 4).

Patient and physician perceived changes in acne severity *vs* depression/anxiety scores: Several negative correlations reached statistical significance in the ITT group but not in the OT group (Table 5). The negative correlations reveal the improvement in psychological symptoms (decreased depression or anxiety scores) along with the decrease in acne severity (magnitude of the improvement perceived by the patient and the physician at a given time point).

Narrative description of the subjects who developed anxiety and/or depression during treatment

Five ITT-treated subjects (13.8% of the ITT group) and two receiving other treatments (8.3% of the OT group patients) developed clinically significant anxiety and/or depression during treatment [χ^2 (1) = 0.06, P = 0.8] (Table 6). In the five subjects of the ITT group, three were females (60%) and two (40%) reported previous symptoms of depression and/or anxiety.

One subject in each group increased their scores in the suicide-related items in the Zung depression scale (items 17 and 19, data not shown). However, because of the very small number of subjects (5 only), an association between gender and previous psychiatric disorders with ITT-induced psychopathology cannot be inferred.

DISCUSSION

This study showed that oral ITT in a fixed-dose schedule of 30 mg/d, compared to a control group of subjects treated with antibiotics or antibacterial agents, was not associated with a significant increase in the frequency of depression or anxiety or in the scores of the corresponding scales up to 12 wk of treatment. This result points to a safe profile of short-term ITT administration in a typical group of non-psychiatric outpatients with acne.

The correlation analysis showed complex results in both treatment groups: On the one hand, only the OT group showed significant correlations (positive ones, mainly in the depression scores) between the physician basal evaluation of acne severity and the psychopathological scales over time (Table 4). This may reveal a poor response to OT because one might have expected a trend toward negative correlations, that is the greater the physician appreciation of the acne severity at baseline, the lower the depression or anxiety scores after successful treatment.

On the other hand, only the ITT-treated patients showed the expected negative correlations between the changes in acne severity observed by the physician and those perceived by the patients (higher scores over time) and the scores in depression and anxiety at each evaluation time point (lower punctuations along time (Table 5). As the correlation data in Table 4 show, this result may imply a better response of the ITT-treated group. However, this is only speculative, because the present investigation was not designed to properly assess the comparative treatment response.

Five ITT-treated subjects developed clinically significant psychopathology: Two patients with pure anxiety, one with pure depression and two with both diagnoses. Personal antecedents of depression or anxiety or significant symptoms at baseline were observed only in two of these five ITT-treated patients. Hence, this small sub-sample of patients does not allow us to explore the predisposing features that favored the development of psychopathology during ITT administration.

The frequency of depression in the ITT group, alone or in combination with anxiety, was 8.3% (3 out of 36 subjects). This value is within the range reported in the literature which is between 1% and 11%[14].

Most research on the psychological deleterious effects of ITT has focused on depression development or aggravation and suicide ideation and/or suicide attempts during treatment[4,5,15-27]. Most recent reviews point to a safe profile of ITT regarding depression and/or suicide[28-31] with the exception of a study conducted by an Australian research group[32]. According to a comprehensive review[4], important methodological differences among the studies would explain the divergent results.

The present research agrees with the conclusion that depression occurs in a minority of ITT-treated patients[4]. Recent studies suggest that these depression-prone patients may be susceptible to other neural side effects of ITT, such as headache[6]. Interestingly, preliminary data suggest that subjects with bipolar disorders may be particularly susceptible of symptom worsening during ITT treatment[33,34]. Since migraine is common in bipolar disorder, particularly in the predominantly depressive type[35], and since psychoses, panic attacks

Table 3 Depression and anxiety levels at baseline and during treatment

	ITT			OT		
			Depression scores			
	Basal	Week 6	Week 12	Basal	Week 6	Week 12
GeDep scale[1]	38.3 ± 2.5	38.1 ± 2.3	36.8 ± 2.3	36.9 ± 3.1	34.9 ± 2.9	34.9 ± 2.9
Zung scale[2]	35.2 ± 1.3	33.8 ± 1.4	34.2 ± 1.4	34.9 ± 1.6	34.6 ± 1.7	35.0 ± 1.7
			Anxiety scores			
	Basal	Week 6	Week 12	Basal	Week 6	Week 12
GegDep scale[3]	43.3 ± 2.4	41.9 ± 2.3	40.8 ± 2.3	40.9 ± 2.9	38.1 ± 2.9	36.8 ± 2.9
Zung scale[4]	37.2 ± 1.1	36.7 ± 1.4	36.6 ± 1.6	34.5 ± 1.4	32.4 ± 1.7	32.8 ± 1.9

Values represent mean ± SD scores in the depression and anxiety scales. [1]Within-subject effect: F (2) = 0.5, P = 0.6; between-subject effect: F (1) = 0.3, P = 0.6; [2]Within-subject effect: F (2) = 0.6, P = 0.6; between-subject effect: F (1) = 0.6, P = 0.5; [3]Within-subject effect: F (2) = 0.5, P = 0.6; between-subject effect: F (1) = 0.9, P = 0.4; [4]Within-subject effect: F (2) = 0.8, P = 0.5; between-subject effect: F (1) = 3.1, P = 0.09. OT: Other treatments; ITT: Isotretinoine.

Table 4 Correlation matrix between the physician assessment of the acne severity at baseline and the depression and anxiety scores before and during treatment

Treatment group		Depression score (baseline)	Depression score (week 6)	Depression score (week 12)	Anxiety score (baseline)	Anxiety score (week 6)	Anxiety score (week 12)
ITT	PAB *vs*	0.1 (0.4)	0.08 (0.6)	0.02 (0.9)	0.1 (0.5)	0.1 (0.4)	0.05 (0.8)
OT	PAB *vs*	0.48 (0.01)[1]	0.5 (0.01)[1]	0.5 (0.01)[1]	0.25 (0.2)	0.39 (0.054)	0.37 (0.07)

PAB: Physician assessment of acne severity at baseline with a scale ranging from minimal = 0 to very severe = 5. Values are the Pearson correlation coefficient and its associated probability in parentheses. [1]Significant association. OT: Other treatments; ITT: Isotretinoine.

Table 5 Correlation matrix of the patient and physician assessments of the acne severity change and the depression and anxiety scores during treatment

Treatment group		Depression score (week 6)	Depression score (week 12)	Anxiety score (week 6)	Anxiety score (week 12)
ITT	Patient evaluation *vs*	-0.31 (0.067)	-0.43 (0.009)[1]	-0.43 (0.01)[1]	-0.38 (0.02)[1]
	Physician evaluation *vs*	-0.28 (0.1)	-0.33 (0.049)[1]	-0.35 (0.03)[1]	-0.32 (0.057)
OT	Patient evaluation *vs*	-0.1 (0.6)	-0.-08 (0.7)	-0.3 (0.9)	0.25 (0.2)
	Physician evaluation *vs*	-0.15 (0.5)	-0.18 (0.4)	-0.03 (0.9)	-0.08 (0.7)

The assessment in acne severity change was conducted with a Likert-type scale ranging from severely worst = 1 to very much improved = 7. Values are the Pearson correlation coefficient and its associated probability in parentheses. [1]Significant association. OT: Other treatments; ITT: Isotretinoine.

Table 6 Demographic and clinical features of the subjects who developed clinically significant depression or anxiety

Treatment	Age (yr)	Gender	Personal history of depression	Personal history of anxiety	Diagnosis before treatment (categorical Zung scales)	Diagnosis during treatment (categorical Zung scales)
ITT	18	M	Yes	Yes	-	Anxiety
ITT	28	M	No	Yes	Anxiety and depression	Anxiety and depression
ITT	25	F	No	No	-	Anxiety and depression
ITT	23	F	Yes	No	-	Anxiety
ITT	23	F	No	No	Depression	Depression
OT	18	M	Yes	Yes	Anxiety and depression	Anxiety and depression
OT	24	M	Yes	No	Depression	Depression

ITT: Isotretinoin; OT: Other treatments; F: Female; M: Male.

and obsessive doubting worsening have also being reported during ITT treatment[8,36-38], the predictive value of headache as a relatively specific marker for ITT-induced depression should be assessed in future studies.

This research field would also benefit from animal studies that would explore the pathways involved in the induction of depression-related behaviors in rodents, such as changes in brain monoamine transmission and corticotrophin-releasing hormone[39,40]. Finally, it is worthwhile mentioning that genetic studies are currently under way to identify genetic polymorphisms in the retinoic receptors that may predispose to or protect from ITT-unintended effects[41].

The present study has the following strengths: A longitudinal evaluation, a control group, the administration of scales for psychopathological assessment that provided categorical and continuous evaluation and the use of locally-developed scales that provided an adequate wording for this specific clinical population. However, it has several limitations: A relatively small sample size and a relatively short period of psychological assessment; the patients were older than those who typically request acne therapy (*i.e.*, adolescents); evaluations were not blind; treatment assignment was not random, and hence, the acne severity was higher in the ITT group than in the control group, and there was no formal scale to assess such a severity.

Further research should take into account that, while random treatment assignment appears problematic from an ethical point of view, semi-quantitative scales for acne severity have been developed[42]. Since severe acne is in many cases a chronic and severe condition and since ITT is often prescribed in doses higher than 30 mg/d and for prolonged periods, long-term studies with high ITT doses are also needed. In those studies, other physical ITT side effects must be assessed. The influence of ethnicity and previous psychiatric disorders on ITT safety should also be examined in further research.

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COMMENTS

Background

Concerns exist about the risk of depression, suicide risk and/or anxiety during isotretinoin (ITT) administration in acne-treated patients.

Research frontiers

Limited information exists about the psychopathology background and the pretreatment mental status of subjects who developed clinically significant depression/anxiety during ITT administration.

Innovations and breakthroughs

Few studies in ITT-treated subjects include an adequate control group, locally-developed psychopathological scales and a pretreatment evaluation. The authors aimed to overcome these limitations in the present study.

Applications

Approximately one out of ten subjects treated for acne either with ITT or antibiotics in the short term may develop clinically-significant depression or anxiety. Subjects at risk for psychopathology must be identified before starting ITT administration.

Terminology

ITT is a vitamin. A-related compound widely used in the treatment of severe cystic acne vulgaris.

Peer-review

Well written manuscript.

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SYSTEMATIC REVIEWS

Internet addiction and problematic Internet use: A systematic review of clinical research

Daria J Kuss, Olatz Lopez-Fernandez

Daria J Kuss, International Gaming Research Unit, Nottingham Trent University, Nottingham NG1 4BU, United Kingdom

Olatz Lopez-Fernandez, Laboratory for Experimental Psychopathology, Psychological Sciences Research Institute, Université Catholique de Louvain, 1348 Louvain-la-Neuve, Belgium

Author contributions: Kuss DJ performed the literature search and both authors contributed to the final write-up of the manuscript.

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Correspondence to: Dr. Daria J Kuss, International Gaming Research Unit, Nottingham Trent University, Burton St, Nottingham NG1 4BU, United Kingdom. daria.kuss@ntu.ac.uk
Telephone: +44-115-8484153
Fax: +44-115-8484153

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Abstract

AIM: To provide a comprehensive overview of clinical studies on the clinical picture of Internet-use related addictions from a holistic perspective. A literature search was conducted using the database Web of Science.

METHODS: Over the last 15 years, the number of Internet users has increased by 1000%, and at the same time, research on addictive Internet use has proliferated. Internet addiction has not yet been understood very well, and research on its etiology and natural history is still in its infancy. In 2013, the American Psychiatric Association included Internet Gaming Disorder in the appendix of the updated version of the Diagnostic and Statistical Manual for Mental Disorders (DSM-5) as condition that requires further research prior to official inclusion in the main manual, with important repercussions for research and treatment. To date, reviews have focused on clinical and treatment studies of Internet addiction and Internet Gaming Disorder. This arguably limits the analysis to a specific diagnosis of a potential disorder that has not yet been officially recognised in the Western world, rather than a comprehensive and inclusive investigation of Internet-use related addictions (including problematic Internet use) more generally.

RESULTS: The systematic literature review identified a total of 46 relevant studies. The included studies used clinical samples, and focused on characteristics of treatment seekers and online addiction treatment. Four main types of clinical research studies were identified, namely research involving (1) treatment seeker characteristics; (2) psychopharmacotherapy; (3) psychological therapy; and (4) combined treatment.

CONCLUSION: A consensus regarding diagnostic criteria and measures is needed to improve reliability across studies and to develop effective and efficient treatment approaches for treatment seekers.

Key words: Internet addiction; Problematic Internet use; Gaming addiction; Internet Gaming Disorder; Clinical studies; Treatment seekers; Treatment; Therapy

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Core tip: Internet addiction has appeared as new mental health concern. To date, reviews have focused on clinical and treatment studies of Internet addiction and Internet Gaming Disorder, limiting the analysis to a specific diagnosis of a potential disorder that has not yet been officially recognised, rather than a comprehensive investigation of Internet-use related addictions (including problematic Internet use) more generally. This systematic literature review outlines and discusses the current empirical literature base for clinical studies of Internet addiction and problematic Internet use. A total of 46 relevant studies on treatment seeker characteristics, psychopharmacotherapy, psychological therapy, and combined treatment were identified.

Kuss DJ, Lopez-Fernandez O. Internet addiction and problematic Internet use: A systematic review of clinical research. *World J Psychiatr* 2016; 6(1): 143-176 Available from: URL: http://www.wjgnet.com/2220-3206/full/v6/i1/143.htm DOI: http://dx.doi.org/10.5498/wjp.v6.i1.143

INTRODUCTION

Over the last 15 years, the number of Internet users has increased by 1000%[1], and at the same time, research on addictive Internet use has proliferated. Internet addiction has not yet been understood very well, and research on its etiology and natural history is still in its infancy[2]. Currently, it is estimated that between 0.8% of young individuals in Italy[3] and 8.8% of Chinese adolescents[4] are affected. The reported higher prevalence rates in China suggest Internet addiction is a serious problem in China, and the country has acknowledged Internet addiction as official disorder in 2008[5].

A comprehensive systematic review of epidemiological research of Internet addiction for the last decade[6] indicated Internet addiction is associated with various risk factors, including sociodemographic variables (including male gender, younger age, and higher family income), Internet use variables (including time spent online, using social and gaming applications), psychosocial factors (including impulsivity, neuroticism, and loneliness), and comorbid symptoms (including depression, anxiety, and psychopathology in general), suggesting these factors contribute to an increased vulnerability for developing Internet-use related problems. Despite the gradually increasing number of studies concerning Internet addiction, classification is a contentious issue as a total of 21 different assessment instruments have been developed to date, and these are currently used to identify Internet addiction in both clinical and normative populations[6]. Conceptualisations vary substantially and include criteria derived from pathological gambling, substance-related addictions and the number of problems experienced. In addition to this, the cut-off points utilised for classification differ significantly, which impedes research and cultural cross-comparisons and limits research reliability.

Increasing research efforts on Internet addiction have led the American Psychiatric Association (APA) to include Internet Gaming Disorder in the appendix of the updated version of the Diagnostic and Statistical Manual for Mental Disorders (DSM-5) in 2013 as condition that requires further research before it can be accepted for inclusion in the main manual[7]. This has resulted in researchers commencing efforts to reach an international consensus for assessing Internet Gaming Disorder using the new DSM-5 approach based on an international expert panel[8]. However, various limitations to this recently proposed "consensus" have been identified, including the lack of a representative international community of experts in the field, the voting method used to arrive at the consensus, the criteria and nosology identified, lack of critical measurement of the disorder and lack of field testing[9]. For the purpose of a comprehensive and inclusive understanding of the potential disorder, in this systematic literature review, Internet addiction will be referred to as encompassing Internet-use related addictions and problematic Internet use, including Internet Gaming Disorder. It is argued that until this concept is understood more fully (including nosology, etiology and diagnostic criteria), limiting our understanding of Internet-use related addictions to Internet gaming-related problems does neither pay sufficient respect to the affected individuals' personal experience nor to the variety of online behaviours that can be engaged in excessively online. For example, other potential online addictions and Internet-use related disorders have been recently reviewed[10], suggesting that limiting a diagnosis to online gaming exclusively misses out many cases of individuals who experience negative consequences and significant impairment due to their Internet use-related behaviours.

For some individuals, their online behaviours are problematic and they require professional help as they cannot cope with their experiences by themselves, suggesting treatment is necessary. Based on in-depth interviews with 20 Internet addiction treatment experts from Europe and North America, Kuss and Griffiths[11] found that in inpatient and outpatient clinical settings, Internet addiction and Internet-use related problems are associated with significant impairment and distress for individuals, which have been emphasised as the criteria demarcating mental disorders[12]. This suggests that in the clinical context, Internet addiction can be viewed as mental disorder requiring professional treatment if the individual presents with significant levels of impairment. Psychotherapists treating the condition indicate the symptoms experienced by the individuals presenting for treatment appear similar to traditional substance-related addictions, including salience,

mood modification, tolerance, withdrawal, conflict and relapse[11]. This view is reflected by patients who seek treatment for their excessive gaming[13].

In 2002, the South Korean government-funded National Information Society Agency has opened the first Internet addiction prevention counselling centre worldwide, and has since developed large-scale projects (including prevention, training, counselling, treatment, and policy formulation) to tackle the pervasive problem of technology overuse[14]. Across the United States and Europe, Internet addiction treatment is not funded by the government, often leaving individuals seeking help either for other primary disorders or through private organisations, although new clinical centres that specialise in treating Internet-use related problems are being developed[15]. Based on the available evidence, recent research furthermore suggests that the best approach to treating Internet addiction is an individual approach, and a combination of psychopharmacotherapy with psychotherapy appears most efficacious[16].

To date, reviews have focused on clinical and treatment studies of Internet addiction[16-19] and Internet Gaming Disorder[2]. This arguably limits the analysis to a specific diagnosis of a potential disorder that has not yet been officially recognised in the Western world, rather than a comprehensive and inclusive investigation of Internet-use related addictions (including problematic Internet use) more generally. Previous reviews relied on overly restrictive inclusion criteria, and this has led to ambiguities in the conceptualisation of the problem, and consequently resulted in limitations regarding both validity and reliability. In order to overcome these problems, the aim of this literature review is to provide a comprehensive overview of clinical studies on the more inclusive clinical picture of Internet-use related addictions from a holistic perspective.

MATERIALS AND METHODS

Between July and August 2015, a literature search was conducted using the database Web of Science. This database is more comprehensive than other commonly used databases, such as PsycINFO or PubMed because it includes various multidisciplinary databases. The following search terms (and their derivatives) were entered: "Internet addict*", "Internet gaming addiction", "gaming addiction", "Internet Gaming Disorder", "compuls* Internet use", "compuls* gam*", "pathological Internet use", "excessive internet use", or "problematic Internet use", and "clinic*", "diagnos*", "treat*, "therap*", or "patient*". Studies were selected based on the following inclusion criteria. Studies had to (1) contain quantitative empirical data; (2) have been published after 2000; (3) include clinical samples and/or clinical interventions for Internet and/or gaming addiction; (4) provide a full-text article (rather than a conference abstract); and (5) be published in English, German, Polish, Spanish, Portuguese, or French as the present authors speak these languages. The initial search yielded 152 results. Following a thorough inspection of the articles' titles and abstracts, the articles that did not meet the inclusion criteria were excluded. The search strategy is presented in Figure 1.

Additional articles were identified through searching the citations in the literature selected, resulting in the inclusion of another eight studies[20-27].

RESULTS

A total of 46 studies met the inclusion criteria. These studies are presented in Table 1. The included studies used clinical samples, and focused on characteristics of treatment seekers and online addiction treatment. Four main types of clinical research studies were identified, namely research involving (1) treatment seeker characteristics; (2) psychopharmacotherapy; (3) psychological therapy; and (4) combined treatment. The results section will outline each of these.

Treatment seeker characteristics

A total of 25 studies[19,26,27,32,43,50,62,72,78,79,93,106,109,111,112,118,124,130,133,143,146,163,164,188,204] investigated the characteristics of treatment seekers. Here, treatment seekers are defined as individuals seeking professional support for online addiction-related problems. The following paragraphs will outline the treatment seekers' sociodemographic characteristics, Internet/gaming addiction measures used to ascertain diagnostic status in the respective studies, differential diagnoses and comorbidities.

Sociodemographic characteristics

In the included studies, sample sizes ranged from a case study of a male in Australia presenting with the problem of generalised pathological Internet use[112] to a total of 1826 clients sampled from 15 inpatient alcohol addiction rehabilitation centres in Germany, of which 71 also presented with Internet addiction and were then compared to a control group of 58 patients treated for alcohol addiction only[188]. Ages ranged from 16 years[112] to a mean age of 30.5 years[72]. The majority of studies used male participants, with one study using female participants only[50]. Most studies included individuals seeking treatment for Internet addiction and/or problematic Internet use in specialised inpatient and outpatient treatment centres. A number of studies included particular samples, such as individuals sampled *via* phone consultations (*i.e.,* including 86% relatives of the affected individuals)[43], patients sampled in alcohol rehabilitation centres[130], patients diagnosed with obsessive compulsive disorder (OCD)[46], and female patients treated for eating disorders[50].

Treatment seekers were sampled from various continents. Within Europe, samples included treatment seekers in Germany[43,78,124,130,133,164,188,197], The Netherlands[50], Italy[26,27,32], and Greece[79]. In North America, a Canadian sample was included[72]. In South America, samples included individuals from Perú[62], Puerto Rico[118], and Brazil[139]. In Western Asia, Turkish individuals were sampled in two studies[143,146], whereas in East Asia, participants were from China[163,204], South

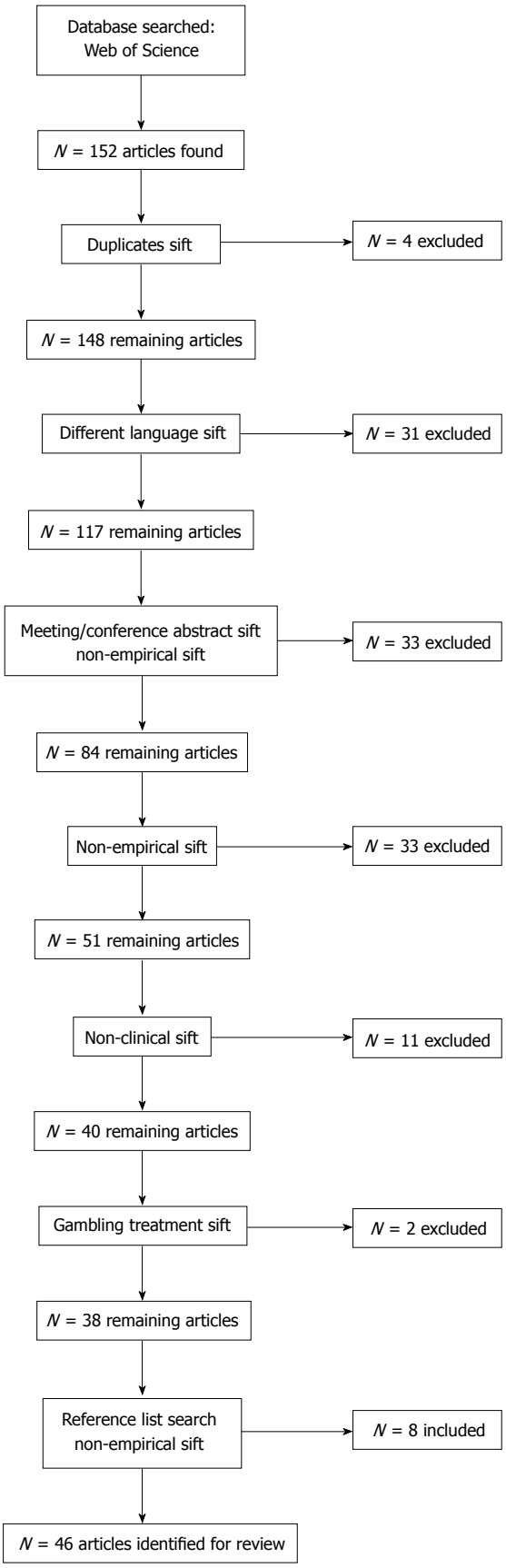

Figure 1 Flow chart displaying the search process.

Korea[93,106,109], and Taiwan[113]. One case study included an Australian adolescent[112].

Internet/gaming addiction

Internet and/or gaming addiction were measured with a number of different psychometric tools in the included studies, sometimes combined with structured clinical interviews. Clinical interviews were explicitly mentioned in the reports of eight studies[32,50,62,93,106,109,164,204], and these consisted mostly of the Structured Clinical Interview for DSM-Ⅳ[64], a semi-structured interview for DSM-Ⅳ Axis Ⅰ diagnoses for mental disorders.

In terms of psychometric measures, in the majority of studies, Young's popular Internet Addiction Test[48], the IAT, was used[26,32,72,93,106,109,118,143,146]. The IAT is a 20-item self-report scale that measures the extent of Internet addiction based on criteria for substance dependence and pathological gambling[51], and includes loss of control, neglecting everyday life, relationships and alternative recreational activities, behavioural and cognitive salience, negative consequences, escapism/mood modification, and deception. Significant problems due to Internet use are identified if individuals score between 70-100 on the test, and frequent problems when they score between 40-69[48]. However, previous research has suggested that across studies, different cut-off scores for the IAT have been used to classify individuals[6], impairing comparisons across studies.

Another popular measure appeared to be the Assessment of Internet and Computer Game Addiction Scale (AICA-S)[44,194], which was used in seven studies[43,78,124,130,133,188,197]. The AICA-S is a 16-item scale and includes questions about the frequency of specific Internet usage, associated negative consequences and the extent to which use is pathological from a diagnostic point of view. Fourteen out of the total sixteen main questions are used to calculate a clinical score, and to distinguish normal from potentially addictive use[211].

Other measures included the Compulsive Internet Use Scale (CIUS)[55], a 14-item unidimensional self-report questionnaire including loss of control, preoccupation (cognitive and behavioural), withdrawal symptoms, coping/mood modification, and conflict (inter- and intrapersonal). The CIUS classification is based on the DSM-Ⅳ TR diagnoses for substance dependence and pathological gambling[12], and was used in one study[50]. Moreover, in one study[79], the Online Cognitions Scale was used[80], which is a 36-item questionnaire that measures cognitions related to problematic Internet use, and includes subscales on loneliness/depression, diminished impulse control, social comfort, and distraction. In another study[113], Chen's Internet Addiction Scale[117] was administered, which is a 26-item self-report measure of core Internet addiction symptoms, including tolerance, compulsive use, withdrawal, and related problems (*i.e.,* negative impact on social activities, interpersonal relationships, physical condition, and time management). Another study[164] used the Internet Addiction Scale[212], as well as a combination of Young's[213] and Beard's[66] Internet addiction criteria, including preoccupation, tolerance, loss of control, withdrawal, overall impairment, deception,

Table 1 Clinical studies reviewed

Study	Aims	Sample and design	Treatment approach	Instruments	Results
Atmaca[28]	To describe a case of problematic Internet use successfully treated with an SSRI-antipsychotic combination	Case report *n* = 1 male 23-yr old single 4th year medical student	SSRI-antipsychotic combination: Citalopram 20 mg/d increased to 40 mg/d within 1 wk, continued for 6 wk; then quetiapine (50 mg/d) added and increased to 200 mg/d within 4 d	SCID-Ⅳ to assess Axis Ⅰ psychiatric comorbidity[29] YBOCS[30,31]	Y-BOCS score decreased from 21 to 7 after treatment Nonessential Internet use decreased from 27 to 7 h/wk; essential Internet use decreased from 4.5 to 3 h/wk Improvement maintained at 4 mo follow-up with the same medication
Bernardi *et al*[32]	To describe a clinical study of individuals with Internet addiction, comorbidities and dissociative symptoms	*n* = 50 adult outpatients self-referred for internet overuse in Italy (age M = 23.3, SD = 1.8 yr) 9 women and 6 men scored ≥ 70 on Internet Addiction Scale; 19 with "possible Internet addiction" (scoring 40-69 on IAT)	N/A	Youngs Internet Addiction Scale IAS[33] Clinical interview DES[34] CGI[35] Sheehan Disability Scale[36] Structured Clinical Interviews for DSM-Ⅳ (SCID Ⅰ and Ⅱ)[37,38] Hamilton Rating Scale for Depression[39] Hamilton Rating Scale for Anxiety[40] Liebowitz Social Anxiety Scale[41] YBOCS[30] CAARS:S[42]	Clinical diagnoses included 14% ADHD, 7% hypomania, 15% generalized anxiety disorder, 15% social anxiety disorder; 7% dysthymia, 7% obsessive compulsive personality disorder, 14% borderline personality disorder, and 7% avoidant personality disorder, 2% binge eating disorder IAD associated with higher perception of family disability and higher Yale-Brown Obsessive Compulsive Severity score Scores for the Dissociative Experience Scale were higher than expected and related to higher obsessive compulsive scores, hours per week on the Internet, and perception of family disability
Beutel *et al*[43]	To present the assessment and clinical characterisation of individuals seeking help for computer and Internet addiction *via* a telephone hotline	*N* = 346 phone consultations (85.8% relatives, 14.2% persons affected) *n* = 131 patients (M = 21.9, SD = 6.6, range 13-47 yr, 96.2% male) Specialised clinic for behavioural addictions in Germany	Telephone consultations First diagnostic interview with expert clinicians	Skala zum Computerspielverhalten [CSV-S (Scale for the Assessment of Pathological Computer Gaming)][44] Symptom-Checklist SCL-90-R[45]	Consultation mainly sought by relatives (86% mothers) 48% reported achievement failure and social isolation, lack of control (38%), family conflicts (33%) 96% of patients (*n* = 131) met criteria for pathological computer gaming
Bipeta *et al*[46]	To compare control subjects with or without Internet addiction with patients with pure obsessive compulsive disorder with or without Internet addiction	*n* = 34 control subjects with or without Internet addiction (age M = 26.9, SD = 6.6 yr) *n* = 38 patients with obsessive compulsive disorder with or without Internet addiction (age M = 27.0, SD = 6.1 yr)	OCD patients treated for 1 year with standard pharmacological treatment for OCD (TAU), received clonazepam, tapered off in three weeks, and an SSRI or clomipramine IA OCD group: 5 received 150-200 mg fluvoxamine/d, 4 received 150-200 mg sertraline/d, 1 received 60 mg fluoxetine/d, 1 received 200 mg clomipramine/d Non-IA OCD group: 8 received 150-300 mg fluvoxamine/d, 5 received 100-200 mg sertraline/d, 11 received 40-80 mg fluoxetine/d, 3 received 150-200 mg clomipramine/d	Youngs Diagnostic Questionnaire[47] IAT[48] Diagnostic and Statistical Manual of Mental Disorders, DSM-Ⅳ (psychiatric interview)[12] BIS-11[49] YBOCS[30]	11 OCD patients (28.95%) diagnosed with IA compared to 3 control subjects OCD group, no difference in OCD scores btw IA/OCD and non-IA/OCD groups IA scores higher in IA/OCD group Treatment improved test scores At 12 mo, 2/11 patients with OCD fulfilled IA criteria

Claes *et al*[50]	To investigate the association among CB, CIU, and reactive/regulative temperament in patients with ED	*n* = 60 female patients with eating disorders in the Netherlands (38.3% with Anorexia nervosa, 6.7% with Anorexia binging-purging type, 26.7% with bulimia nervosa, and 28.3% with Eating Disorder not otherwise specified; age range 15-57 yr, mean age = 27.8, SD = 9.8 yr)	N/A	DSM-Ⅳ, standardised clinical interview[51] EDI-2[52,53] CBS[54] Dutch Compulsive Internet Use Scale[55] BIS/BAS scales[56,57] DAPP[58,59] Adult Temperament Questionnaire-Short Form[60,61]	Positive association btw CB and CIU, emotional lability, excitement seeking, lack of effortful control (lack of inhibitory and lack of activation control) 11.7% of CB patients with IA No significant differences between ED subtypes regarding CIU
Cruzado Díaz *et al*[62]	To describe clinical and epidemiological characteristics of inpatients in a clinical centre in Perú between 2001-2006	*n* = 30 patients with "IA" 90% devoted themselves to online games) in Perú All single males from 13 to 28 yr old (M = 18.3, SD = 3.8), 63.3% with secondary education completed and 66.7% dropped out Descriptive, retrospective and transversal study	N/A	Reviewed 30 clinical registers through FEIA[63], a semi-structured instrument for psychiatric evaluation applied to clinical histories Patients completed a brief survey through an interview regarding information about their Internet use and online behaviours	Patient characteristics: Young age (18.3 ± 3.8 yr old) Extensive daily Internet use (50% remained online for more than 6 h/d) Primary Internet use: Online gaming (43.3% excessive gaming and 6.7% excessive gambling) Comorbidities (DSM-Ⅳ): High frequency of psychopathic behaviours (antisocial personality traits: 40%), 56.7% affective disorders (30% major depression and 26.7% dysthymia), 26.7% other addictions (13.3% gambling, 10% alcohol, 10% marihuana, 6.7% nicotine and 3.3% cocaine), 16.7% antisocial disorders (13.3% ADHD, social phobia 10% and 3.3% dysmorphic corporal disorder)
DellOsso *et al*[20]	To assess the safety and efficacy of escitalopram in IC-IUD using a double-blind placebo-controlled trial	*n* = 19 adult subjects (12 men, mean age = 38.5, SD = 12.0 yr) with IC-IUD (as primary disorder) 19 wk prospective trial with 2 consecutive phases: 10 wk treatment phase (*n* = 17, 11 men, mean age = 37.5, SD = 12.0 yr = and 9 wk randomised double-blind placebo controlled trial (*n* = 14, 10 men, mean age = 40.0, SD = 11.5 yr)	Escitalopram started at 10 mg/d, increased and maintained at 20 mg/d for 10 wk Subsequently, participants randomly assigned to placebo or escitalopram for 9 wk	Structured Clinical Interview for DSM-Ⅳ Axis I[64] Time spent in non-essential Internet use (hours/wk) CGI-I[35] BIS[49] IC-IUD version of YBOCS[30]	Following double-blind phase, there were no significant differences in weekly non-essential Internet use and overall clinical response between treatment and placebo group Side effects: Fatigue and sexual side effects in treatment, but not placebo group

Du *et al*[65]	To evaluate the therapeutic effectiveness of group CBT for Internet addiction in adolescents	*n* = 56 adolescents with IA (age range 12-17 yr) *n* = 32 active treatment group (28 male, mean age = 15.4, SD = 1.7 yr) *n* = 24 clinical control group (17 male, mean age = 16.6, SD = 1.2 yr)	Group cognitive behavioural therapy: Active treatment group: 8 1.5-2 h sessions of multimodal school-based group CBT with 6-10 students/group run by two child and adolescent psychiatrists (topics: Control, communication, Internet awareness, cessation techniques); group CB parent training; psychoeducation delivered to teachers Clinical control group: No intervention	Beards Diagnostic Questionnaire for Internet addiction[66] Internet Overuse Self-Rating Scale[67,68] Time Management Disposition Scale[69] Strength and Difficulties Questionnaire (Chinese edition)[70] SCARED[71]	Internet use decreased in both groups Only treatment group had improved time management skills and better emotional, cognitive and behavioural symptoms
Dufour *et al*[72]	To describe the sociodemographic characteristics of Internet addicts in a CDR, and to document their problems associated with other dependencies (alcohol, drugs, game practices), self-esteem, depression and anxiety	*n* = 57 Internet addiction treatment seekers (88.4% males, 11.6% females; age range = 18-62 yr (M = 30.5, SD = 11.8 yr). Canada	N/A	IAT[48,73] Becks Anxiety inventory[74] Becks Depression inventory[75] DÉBA-Alcohol/Drugs/Gaming[76] Self-esteem[77]	88% of Internet addicts were male, with a mean age of 30, living with their parents with low income M = 65 h of Internet use per week: 57.8% MMORPGs, 35,1% video streaming, and 29.8% chat rooms Rosenberg test: 66.6% weak and very weak self-esteem; Depression in only 3.5% and anxiety in 7.5% 45.6% received pharmacological treatment for mental disorders (psychotropic) and 33.3% had a chronic physical problem
Duven *et al*[78]	To investigate whether an enhanced motivational attention or tolerance effects are reported by patients with IGD	*n* = 27 male clinical sample from specialised behavioural addiction centre in Germany (*n* = 14 with IGD, *n* = 13 casual computer gamers) Semi-natural EEG designed with participants playing a computer game during the recording of event-related potentials to assess reward processing	N/A	AICA-S[44] SCL-90-R[45]	Attenuated P300 for patients with IGD in response to rewards relative to a control group Prolonged N100 latency and increased N100 amplitude, suggesting tolerance during computer game play, and gaming reward attention uses more cognitive capacity in patients
Floros *et al*[79]	To assess the comorbidity of IAD with other mental disorders in a clinical sample	*n* = 50 clinical sample of college students presenting for treatment of IAD in Greece (39 males, mean age = 21.0, SD = 3.2 yr; 11 females, mean age = 22.6, SD = 4.5 yr) Cross-sectional study	N/A	OCS[80] DSQ[81] ZKPQ[82,83] SCL-90[84,85]	25/50 presented with comorbidity of another Axis I disorder (10% with major depression, 5% with dysthymia and psychotic disorders, respectively), and 38% (19/50) with a concurrent Axis II personality disorder (22% with narcissistic, and 10% with borderline disorder) The majority of Axis I disorders (51.85%) were reported before IAD onset, 33.3% after onset
Ge *et al*[86]	To investigate the association between P300 event-related potential and IAD	*n* = 41 IAD subjects (21 males, age M = 32.5, SD = 3.2 yr) *n* = 48 volunteers (25 males, age M = 31.3, SD = 10.5 yr) Experimental task	CBT	Standard auditory oddball task using American Nicolet BRAVO Instrument	IA individuals had longer P300 latencies, but similar P300 amplitudes compared to controls Following treatment, P300 latencies decreased significantly, suggesting cognitive function deficits associated with IAD can be ameliorated with CBT

Han and Renshaw[21]	To test whether bupropion treatment reduces the severity of EOP and MDD	*n* = 50 male subjects with EOP and MDD (aged 13-45 yr)	Random allocation to either bupropion and EDU group or placebo and EDU group	Structured Clinical Interview for DSM-Ⅳ[64]	During active treatment period, Internet addiction, gaming, and depression decreased relative to placebo group
		n = 25 treatment group (mean age = 21.2, SD = 8.0 yr, range = 13-42) *n* = 25 placebo group (mean age = 19.1, SD = 6.2 yr, range = 13-39)	12-wk treatment (8 wk active treatment phase and 4-wk post treatment follow-up period)	Youngs Internet Addiction Scale[87,88]	During follow-up, bupropion-associated reductions in gaming persisted, while depressive symptoms recurred
		Randomised controlled double-blind clinical trial	150 mg/d Bupropion SR given and increased to 300 mg/d during first week	Becks Depression Inventory[89]	
Han *et al*[24]	To test the effects of bupropion sustained release treatment on brain activity for Internet video game addicts	*n* = 11 IAG (IAG; mean age = 21.5, SD = 5.6 yr; mean craving score = 5.5, SD = 1.0; mean playing time = 6.5, SD = 2.5 h/d; mean YIAS score = 71.2, SD = 9.4)	Placebo group started with one pill and then raised to two pills	Structured Clinical Interview for DSM-Ⅳ[64]	Bupropion sustained release treatment works for IAG in a similar way as it works for patients with substance dependence
		n = 8 HC (HC; mean age = 11.8, SD = 2.1 yr; mean craving score = 3.9, SD = 1.1; mean Internet use = 1.9, SD = 0.6 h/d; mean YIAS score = 27.1, SD = 5.3) in South Korea	Buproprion sustained release treatment: 6 wk	Beck Depression Inventory[89] Youngs Internet Addiction Scale[87]	During exposure to game cues, IAG had more brain activation in left occipital lobe cuneus, left dorsolateral prefrontal cortex, left parahippocampal gyrus relative to HC
		Experimental design	Participants underwent 6 wk of bupropion sustained release treatment (150 mg/d for first week, 300 mg/d afterwards)	Craving for Internet video game play: 7-point visual analogue scale Brain activity measured at baseline and after treatment using 1.5 Tesla Espree fMRI scanner	After treatment, craving, play time, cue-induced brain activity decreased in IAG
Han *et al*[22]	To assess the effect of methylphenidate on Internet video game play in children with ADHD	*n* = 62 children (52 males, mean age = 9.3, SD = 2.2 yr, range = 8.12), drug-naïve, diagnosed with ADHD, and Internet video game players in South Korea	Treatment with Concerta (OROS methylphenidate HCI, South Korea) Initial dosage: 18 mg/d, and maintenance dosage individually adjusted based on changes in clinical symptoms and weight	YIAS-K[87,88] Korean DuPaul's ADHD Rating Scale[90,91] Visual Continuous Performance Test using the Computerised Neurocognitive Function Test[92]	Following treatment, Internet addiction and Internet use decreased Changes in IA between baseline and treatment completion correlated with changes in ADHD, and omission errors from the Visual Continuous Performance Test
Hwang *et al*[93]	To directly compare patients with IA to patients with AD regarding impulsiveness, anger expression, and mood	*n* = 30 patients with IA (mean age = 22.7, SD = 6.7 yr)	N/A	Korean version of Youngs IAT[48,94]	IA and AD groups showed lower agreeableness and higher neuroticism, impulsivity, and anger expression compared to the HC group (all related to aggression)
		n = 30 patients with AD (mean age = 30.0, SD = 5.9 yr)		SCID[64]	Addiction groups had lower extraversion, openness to experience, and conscientiousness, were more depressive and anxious than HCs
		n = 30 HCs (HCs, mean age = 25.3, SD = 2.8 yr) Outpatient clinic in South Korea		Alcohol Use Disorder Identification Test-Korean version[95] Korean version of the NEO-PI-R[96,97] Korean version of the BIS-11[98,99] Korean version of the STAXI-K[100,101]	Severity of IA and AD positively correlated with these symptoms

Kim[23]	To examine the effect of a reality therapy (R/T) group counselling programme for Internet addiction and self-esteem	*n* = 25 university students in South Korea (20 males, mean age = 24.2 yr) Randomised controlled trial/quasi- experimental design	Treatment group (*n* = 13, 10 males): Participated in R/T group counselling programme, 2 60-90 min sessions/wk for 5 consecutive weeks (with the purpose of taking control and changing thinking and behaviours) Control group (*n* = 12, 10 males): No treatment	K-IAS[102] CSEI[103]	Treatment programme reduced addiction level and increased self-esteem
Kim *et al*[25]	To evaluate the efficacy of CBT combined with bupropion for treating POGP in adolescents with MDD	*n* = 65 adolescents with MDD and POGP in South Korea (aged 13-18 yr) Prospective trial	*n* = 32 CBT group (medication and CBT): 8 wk intervention; 159 mg bupropion/d for 1 wk, then 300 mg/d for 7 wk; participated in 8 session weekly group CBT; weekly 10 min interviews *n* = 33 clinical control group (medication only, as above)	BDI[89] BAI[74] YIAS[87,88] Modified-School Problematic Behaviour Scale[104] Modified Students Life Satisfaction scale[105]	Internet addiction decreased and life satisfaction increased in CBT and medication group relative to medication only group, but no changes in depression Anxiety increased in medicated group
Kim *et al*[106]	To investigate the value of Youngs IAT for subjects diagnosed with Internet addiction	*n* = 52 individuals presenting with Internet addiction at university hospital in South Korea (47 males; mean age = 21.7, SD = 7.1 yr, range: 11-38)	N/A	Clinical interview Youngs IAT[107,108] Classification of IA severity *via* DSM-Ⅳ-TR[12]	Samples mean IAT score below cut-off (70) IAT detected only 42% of sample as having Internet addiction No significant differences in IAT scores between mild, moderate and severe Internet addition found No association between IAT scores and Internet addiction duration of illness found IAT has limited clinical utility for evaluating IA severity
Kim *et al*[109]	To compare patients with IGD with patients with AUD and HC regarding resting-state ReHo	*n* = 45 males seeking treatment in South Korea *n* = 16 IGD patients (mean age = 21.6, SD = 5.9 yr) *n* = 14 AUD patients (mean age = 28.6, SD = 5.9 yr) *n* = 15 HCs (mean age = 25.4, SD = 5.9 yr)	N/A	Youngs IAT[87] SCID[64] AUDIT-K[110] BDI[89] BAI[74] BIS-11[111] FMRI resting data acquired via Philips Achieva 3-T MRI scanner using standard whole-head coil, obtaining 180 T2 weighted EPI volumes in each of 35 axial planes parallel to anterior and posterior commissures	Significantly increased ReHo in PCC of the IGD and AUD groups Decreased ReHo in right STG of IGD, compared with AUD and HC groups Decreased ReHo in anterior cingulate cortex of AUD patients Internet addiction severity positively correlated with ReHo in medial frontal cortex, precuneus/ PCC, and left ITC in IGD Impulsivity negatively correlated with ReHo in left ITC in IGD Increased ReHo in PCC: Neurobiological feature of IGD and AUD Reduced ReHo in STG: Neurobiological marker for IGD specifically relative to AUD and HCs
King *et al*[112]	To present a case study of an individual with GPIU	*n* = 1, 16-yr old male in Australia Case study	N/A	N/A	PIU identified due to: (1) use of several different Internet functions; (2) social isolation; (3) procrastination and time-wasting tendencies Problems unlikely to have occurred without the Internet

Ko *et al*[113]	To evaluate the diagnostic validity of IGD criteria, and to determine the cut-off point for IGD in DSM-5	*n* = 225 adults in Taiwan (*n* = 75 individuals with IGD (63 males, mean age = 23.4, SD = 2.6 yr), no IGD (63 males, mean age = 22.9, SD = 2.5 yr), and IGD in remission (63 males, mean age = 23.8, SD = 2.9 yr), respectively)	N/A	Diagnostic interview based on DSM-5 IGD criteria[7] DC-IA-C[114] Chinese version of the MINI[115] QGU-B[116] CIAS[117]	Diagnostic accuracy of DSM-5 IGD items between 77.3% and 94.7% (except for deceiving and escape), and differentiated IGD from remitted individuals Meeting ⩾ 5 IGD criteria: Best cut-off point to differentiate IGD from non-IGD and remitted individuals
Liberatore *et al*[118]	To describe the prevalence of IA in a clinical sample of Latino adolescents receiving ambulatory psychiatric treatment	*n* = 71 adolescent patients in Puerto Rico (39 males, aged 13-17 yr), 39.4% diagnosed with disruptive disorder, 31.0% with mood disorder, 19.7% with mood and disruptive disorder	N/A	Spanish version of the Internet Addiction Test (IAT)[87]	Sample did not involve any cases of severe IA 71.8% of the sample had no IA problem 11.6% discussed Internet use with therapists IA correlated with mood disorders
Liu *et al*[119]	To test the effectiveness and underlying MFGT	*n* = 92 (46 adolescents with 12-18 yr old, and 46 parents, aged 35-46 yr old) 2 groups: 1 experimental (EG; MFGT adolescents and parents) and 1 control (CG; waiting-list similar adolescents and parents) EG: Adolescents: 17 males and 4 females (age: M = 15, SD = 1.73); Parents: 5 males and 16 females (age: M = 40.9, SD = 2.85) CG: Adolescents: 21 males and 4 females (age: M = 15.7, SD = 1.2); Parents: Idem to EG (no sign. Diff). China Quasi-experimental design	MFGT is a new approach to treat Internet addiction (IA) behaviours that has not been tested before MFGT = group therapy for families, both adults and adolescents that have the same problem (IA) Advantage: Peer group (support and learn from peer confrontation) Transference reactions occur within and between families	Structured questionnaires at pre-test (T1), post-test (T2) and follow-up (T3): Adolescents scales: Adolescent Pathological Internet Use Scale APIUS[120] Parents scales: Closeness to Parents[121] Parent-Adolescent Communication Scale[122] College Students Psychological Needs and Fulfillment Scale[123]	Significantly decreased IA in EG at T2 and maintained in T3 (adolescents IA rate dropped from 100% at baseline to 4.8% after intervention, then remained at 11.1%) Significantly better reports in the EG from adolescents and parents compared with those in the CG Underlying mechanism of less IA was partially explained by adolescent satisfaction of their psychological needs and improved parent-adolescent communication and closeness
Müller *et al*[124]	To characterize German treatment seekers and to determine the diagnostic accuracy of a self-report scale for IA	*n* = 290 mostly male (93.8%) treatment seekers between 18 and 64 yr (M = 26.4, SD = 8.22) Germany	Treatment of behavioural addictions Non-experimental design	SCL-90R[125] PHQ[126] GAD-7[127] CDS-2[128] AICA-S[129]	71% met clinical IA diagnosis Displayed higher levels of psychopathology, especially depressive and dissociative symptoms Half met criteria for one further psychiatric disorder, especially depression Level of functioning decreased in all domains AICA-S showed good psychometric properties and satisfying diagnostic accuracy (sensitivity: 80.5%; specificity: 82.4%)
Müller *et al*[130]	To compare personality profiles of a sample of patients in different rehabilitation centres	IA group: 70 male patients with an addiction disorder that additionally met the criteria for IA; M =29.3 yr (SD = 10.66; range 16-64) AD group: 48 male patients suffering from AD; M = 31.7 yr; SD = 9.18; range 17-65) Germany	N/A Non-experimental design	Computer game Addiction (AICA-S)[129] NEO-FFI[131] BDI-II[132]	Patients with comorbid IA can be discriminated from other patients by higher neuroticism and lower extraversion and lower conscientiousness After controlling for depressive symptoms, lower conscientiousness turned out to be a disorder-specific risk factor

Müller *et al*[133]	To evaluate the relationships between personality traits and IGD	*n* = 404 males aged 16 yr and above 4 groups: IGD group: 115 patients with IGD Clinical CG: 74 controls seeking treatment for IGD, but not diagnosable Gambling group: 115 gambling patients Healthy CG: 93 individuals with regular or intense use of online games Germany	N/A Experimental design: Characteristics of people selected for assigning them to two groups, non-random allocation	AICA-S[44] AICA-C[134] Berlin Inventory for Gambling[135] NEO Five-Factor Inventory[131]	IGD associated with higher neuroticism, decreased conscientiousness and low extraversion The comparisons to pathological gamblers indicate that low conscientiousness and low extraversion in particular are characteristics of IGD Etiopathological model proposed for addictive online gaming
Park *et al*[136]	To examine the effectiveness of treating an Internet-addicted young adult suffering from interpersonal problems based on the MRI interactional model and Murray Bowen's family systems theory	1 family case study consisting of husband (age 50), wife (age 50), 2 sons (ages 22, 23), older son with Internet addiction and interpersonal problems South Korea		Comparative analysis method Miles and Huberman's matrix and network[137]	Characteristics of the parents family of origin and dysfunctional communication pattern associated with interpersonal problems revealed by participants Both the MRI model and Bowen's family systems theory produced effective treatments
Poddar *et al*[138]	To describe a pilot intervention using MET and CBT principles to treat IGD in an adolescent	*n* = 1 14-yr-old boy India Case study	Initial therapy session: Rapport building with patient, detailed interview, primary case formulation Subsequent sessions: Psychoeducation, cost/benefit analysis of behaviour (motivation level improved) Progressive muscle relaxation because gaming urge accompanied by physiological/emotional arousal Subsequently: Game addiction assessment, contract for behaviour modification (reduce gaming time, increase other activities) Tokens introduced as positive reinforcement Less time spent gaming on weekdays, but excess on weekends Patient recorded Thoughts, Emotions and Behaviors (TE and B) contributing to gaming (result: Gaming due to boredom) Non-gaming behaviour reinforced *via* scooty rides	IQ ESDST, BVMGT, and TAT IAT	IGD due to child neglect and boredom, consolidated by subsequent negative reinforcements Individual interventions encouraged as there are varied antecedents and consequences for IGD development MET-CBT principles for IGD resulted in improvement Therapy terminated when gains had consolidated Good exam scores achieved Weekend gaming times reduced IAT score reduced to 48 (from 83)

Santos *et al*[139]	To describe a treatment of a patient with PD, OCD (both anxiety disorders) and IA involving pharmacotherapy and CBT and test its efficacy	Case report *n* = 1 24-yr-old Caucasian woman A patient with PD, OCD and IA Brazil	Pharmacotherapy and CBT CBT 1x/week for 10 wk Pharmacotherapy [clonazepam (0.5 mg) and sertraline (50 mg) once daily] Both (pharmaco and CBT) started together CBT focus: Teach patient how to deal with anxiety and internet use (*i.e.*, breathing retraining with diaphragmatic breathing exercise, education about PD and OCD symptoms and internet use, time management, identifying PIU triggers, changing habits, cognitive restructuring, exposure and response prevention, social support promotion, building alternative activities, functional internet use promotion)	Hamilton Anxiety Scale (HAMA-A)[40] Hamilton Depression Scale (HAM-D)[39] Chambless BSQ[140] Bandelow PA[141] IAT CGI[142]	Treatment effective for anxiety and IA
Senormanci *et al*[143]	To investigate the attachment styles and family functioning of patients with IA	*n* = 60 2 groups: EG: 30 male patients with IA [age: M = 21.6 (18-20) yr] CG: 30 healthy males without IA Non-experimental	N/A	IAT[48] BDI[89] Experiences in Close Relationships Questionnaire-r [144] Family Assessment Device[145]	Patients with IA had higher BDI and higher attachment anxiety sub-scores on the ECR-r compared with those in the CG IA patients evaluated their family functioning as more negative and reported problems in every aspect addressed by the FAD Scores on the FAD behaviour control, affective responsiveness, and problem-solving subscales (and on the FAD communication, roles, and general functioning subscales) significantly higher in patients compared with CG
Senormanci *et al*[146]	To determine the predictor effect of depression, loneliness, anger and interpersonal relationship styles for IA in patients diagnosed with IA	*n* = 40 male IA patients with at least 18-yr-old Turkey	N/A	IAT[48] BDI[89] STAXI[100] UCLA Loneliness Scale[147] IRSQ, subscale "Contributing and inhibiting styles"[148]	Duration of Internet use (hours/day) and STAXI anger in subscale predicted IA. Although the duration is not adequate for IA diagnosis, it predicts IA It is helpful for clinicians to regulate the hours of Internet use for patients with excessive or uncontrolled internet use Psychiatric treatments for expressing anger and therapies focussing on emotion validation may be useful

Shek *et al*[149]	To described an indigenous multi-level counselling programme designed for young people with IA problems based on the responses of clients	*n* = 59 58 male and 1 female Most in early adolescence (aged 11-15 yr; *n* = 29) and late adolescence (aged 16-18 yr; *n* = 27), while 3 were over 18 China	Indigenous multilevel counselling program designed to provide services for young people with Internet addictive behaviour in Hong Kong: (1) Emphasis on controlled and healthy use of the Internet; (2) Understanding the change process in adolescents with Internet addiction behaviour; (3) Utilization of motivational interviewing model; (4) Adoption of a family perspective; (5) Multi-level counselling model; (6) Utilization of case work and group work	3 versions of IA Young's assessment tools[150]: 10-item, 8-item and 7-item measures[151-153] Goldberg's framework[154] Chinese Internet Addiction Scale (CIA-Goldberg) Items for assessing beliefs and behaviours for using Internet: 7 items from Computer Use Survey[155] 6 items from OCS[80] 6 items from Internet Addiction-Related Perceptions and Attitudes Seale[156] 2 items from IAD-Related Experience Scale[157] 33-item C-FAI developed[158] Chinese Purpose in Life Questionnaire[159] Chinese Beck Depression Inventory[160] Chinese Hopelessness Seale[161] Chinese Rosenberg Self-Esteem Seale[162]	The outcome evaluation, pretest and posttest data showed IA problems decreased after joining programme Slight positive changes in parenting attributes Participants subjectively perceived the programme was helpful
Tao *et al*[163]	To develop diagnostic criteria for IAD and to evaluate the validity of proposed diagnostic criteria for discriminating non-dependent from dependent Internet use in the general population	3 stages: Criteria development and item testing; criterion-related validity testing; global clinical impression and criteria evaluation; Stage 1: *n* = 110 patients with IA in SG, M = 17.9 SD = 2.9 yr (range: 12-30 yr), 91.8% (*n* = 101) males; 408 patients in IA in TG, M = 17.6, SD = 2.7 yr (range: 12-27 yr), 92.6% (*n* = 378) male; Stage 2: *n* = 405; Stage 3: *n* = 150 (M = 17.7, SD = 2.8, (92.7% males) China	N/A	N/A: Authors developed the proposed Internet addiction diagnostic criteria, which have been one of the main sources for the APAs IGD criteria	Proposed Internet addiction diagnostic criteria: Symptom criterion (7 clinical IAD symptoms), clinically significant impairment criterion (functional and psychosocial impairments), course criterion (duration of addiction lasting at least 3 mo, with at least 6 h of non-essential Internet use per day) and exclusion criterion (dependency attributed to psychotic disorders) Diagnostic score of 2 + 1, where first 2 symptoms (preoccupation and withdrawal symptoms) and min. 1/5 other symptoms (tolerance, lack of control, continued excessive use despite knowledge of negative effects/ affects, loss of interests excluding Internet, and Internet use to escape or relieve a dysphoric mood) was established Inter-rater reliability: 98%

Te Wildt *et al*[164]	To examine the question whether the dependent use of the Internet can be understood as an impulse control disorder, an addiction or as a symptom of other psychiatric conditions	EG: *n* = 25 patients (76% male, M = 29.36 yr, SD = 10.76) CG: Matched for age (M = 29.48; SD = 9.56), sex (76% males) and school education, and similar level of intelligence	2 groups matched: The EG and CG Non-experimental design	Preliminary telephone interview to test inclusion criteria with Young's and Beard's IA criteria[48,66] Statistical Clinical Interview for DSM-IV[64] German Internet Addiction Scale ISS[165] German version of the Barratt Impulsiveness Scale BIS[49] Derogatis Symptom Checklist (SCL-90-R)[166,167] BDI[89,168] DES[169,170] SOC[171,172] IIP-D[173,174]	Compared to controls, patient group presented significantly higher levels of depression (BDI), impulsivity (BIS) and dissociation (DES) PIU shares common psychopathological features and comorbidities with substance-related disorders Should be viewed as diagnostic entity in itself within a spectrum of behavioural and substance dependencies
Tonioni *et al*[26]	To test whether patients with IA present different psychological symptoms, temperamental traits, coping strategies and relational patterns relative to patients with PG	Two clinical groups: 31 IA patients (30 males) and 11 PG patients (10 males) and a control group (38 healthy subjects; 36 males) matched with the clinical groups for gender and age were enrolled	N/A	IAT[48] Hamilton Anxiety Rating Scale[40] Hamilton Depression Scale[39] Global Assessment of Functioning[112] Snaith-Hamilton Pleasure Scale[175] Temperament and Character Inventory-Revised[176] Coping Orientation to Problems Experienced[177] Inventory of Parent and Peer Attachment[178]	IA and PG had higher scores than control group on depression, anxiety and global functioning IA patients had higher mental and behavioural disengagement associated with an important interpersonal impairment relative to PG patients IA and PG groups used impulsive coping, and had socio-emotional impairment
Tonioni *et al*[27]	To investigate psychopathological symptoms, behaviours and hours spent online in patients with IAD	*n* = 86: 21 clinical patients in hospital-based psychiatric IAD service (mean age=24, SD = 11 yr); 65 control subjects	N/A	Internet addiction interview[47] IAT[179] Symptom Checklist-90-Revised[125]	IAD patients had significantly higher scores on IAT relative to controls Only item 7 (how often do you check your e-mail before something else that you need to do?) showed a significant inverse trend SCL-90-R anxiety and depression scores and IAT item 19 (How often do you choose to spend more time online over going out with others?) positively correlated with weekly online hours in IAD patients

van Rooij *et al*[180]	To evaluate the pilot treatment for IA created for the Dutch care organization (to explore the possibility of using an existing CBT and MI based treatment programme (lifestyle training) from therapists experiences with 12 Internet addicts	*n* = 12 Internet addicts and *n* = 5 therapists treating them The Netherlands	Treatment: A manual-based CBT Standard Lifestyle Training programme, a manual-based treatment programme[181,182] Therapy combines CBT and MI[183,184] Focuses on eliciting and strengthening motivation to change, choosing a treatment goal, gaining self-control, relapse prevention, and coping skills training[185,186] 10 outpatient sessions of 45 min each, with 7 of these taking place within a period of 10 wk, the remaining 3 within a period of 3 mo Each session: Introduction, evaluation of current status, discussing homework, explaining theme of the day, practicing a skill, receiving homework, and finally closing the session	Data sources: (1) Session Reports; (2) Case Review Meeting Minutes; (3) Questionnaires: Compulsive Internet Use Scale (CIUS)[55] Brief Situational Confidence questionnaire[187]	Therapists report programme (originally used for substance dependence and pathological gambling) fits problem of Internet addiction well Interventions focused on controlling and reducing Internet use, and involved expanding (real life) social contacts, regaining proper daily structure, constructive use of free time, and reframing beliefs Therapist report: Treatment achieved progress for all 12 treated patients Patient report: Satisfaction with treatment and behavioural improvements
Wölfling *et al*[188]	To investigate whether IA is an issue in patients in addiction treatment	*n* = 1826 clients in impatient centres Male patients meeting criteria for comorbid IA (EG; *n* = 71) compared with a matched control group of male patients treated for alcohol addiction without addictive Internet use (CG; *n* = 58) Germany	N/A	Internet and Computer Game Addiction (AICA-S)[189,190] Symptom Checklist 90R (SCL-90-R)[191] PHQ[126] GAD-7[127]	Comorbid IA associated with higher levels of psychosocial symptoms, especially depression, obsessive-compulsive symptoms, and interpersonal sensitivity IA patients meet criteria for additional mental disorders more frequently and display higher rates of psychiatric symptoms, especially depression, and might be in need of additional therapeutic treatment

Wölfling *et al*[192]	To test the effects of a standardized CBT programme for IA	*n* = 42 patients with IA, all male from 16-yr-old (M = 26.1, SD = 6.60, range: 18-47)	Short-Term outpatient Treatment for Internet and Computer Game Addiction STICA (127) based on CBT techniques known from treatment programmes of other forms of addictive behaviour, consisting of 15 group sessions and additional 8 individual therapy sessions Individual sessions dealt with individual contents; group sessions followed clear thematic structure: First third of programme: Main themes about development of individual therapy aims, identification of Internet application associated with symptoms of IA, conducting holistic diagnostic investigation of psychopathological symptoms, deficits, resources, and comorbid disorders Motivational techniques applied to enhance patients intention to cut down dysfunctional behaviour Second third: Psychoeducation elements; deepened Internet use behaviour analysis (focusing on triggers and patient reactions on cognitive, emotional, psychophysiological, and behavioural levels in that situation (SORKC scheme)[193] for development of a personalized model of IA for each patient based on interaction between online application, predisposing and maintaining factors of the patient (*e.g.*, personality traits) and the patients social environment Last stage: Situations with heightened craving for getting online further specified and strategies to prevent relapse developed	Inclusion criteria: AICA-S[193,194] Standardized clinical interview of IA (AICA-C; Checklist for the Assessment of Internet and Computer Game Addiction)[132] GSE[195] NEO Five-Factor Inventory[131] Symptom Checklist 90R[196]	70.3% of patients completed therapy After treatment, symptoms of IA decreased significantly Psychopathological symptoms and associated psychosocial problems decreased

Wölfling *et al*[197]	To investigate the occurrence of BSD in patients with excessive Internet use and IA	*n* = 368 treatment seekers with excessive to addictive Internet use screened for bipolar spectrum disorders Germany	N/A	AICA-S[194] BSD assessed using MDQ[198] SCL-90R[199,200]	Comorbid BSD more frequent in patients meeting criteria for IA (30.9%) than among excessive users (5.6%) This subgroup showed heightened psychopathological symptoms, including substance use disorders, affective disorders and personality disorders Further differences were found regarding frequency of Internet use regarding social networking sites and online-pornography in patients with BSD who engage more frequently Patients with IA have heightened probability for meeting BSD criteria Recommendation: Implement BSD screening in patients presenting with IA
Young[201]	To investigate the efficacy of using CBT with Internet addicts	*n* = 114 Internet addicts in treatment (42% women (mean age = 38; men mean age = 46)	Sessions conducted between client and principle investigator Initial sessions gathered familial background, nature of presenting problem, its onset and severity CBT utilized to address presenting symptoms related to computer use, specifically abstinence from problematic online applications and strategies to control online use Counselling also focused on behavioural issues or other underlying factors contributing to online abuse, such as marital discord, job burnout, problems with co-workers, and academic troubles, depending on respective client	IAT[48] Self-devised Client Outcome Questionnaire administered after 3rd, 8th, and 12th online session, and at 6 mo follow-up: 12 items regarding clients behaviour patterns and treatment successes during counselling process; questions rated how effective counselling was at helping clients achieve targeted treatment goals associated with Internet addiction recovery; questions assessed motivation to quit Internet abuse, ability to control online use, engagement in offline activities, improved relationship functioning, and improved offline sexual functioning (if applicable)	Preliminary analyses indicated most clients managed their presenting complaints by the eighth session Symptom management sustained at 6-mo follow-up

Young[202]	To test a specialized form of CBT, CBT-IA	*n* = 128 clients to measure treatment outcomes using CBT-IA (65% male; age range: 22-56 yr)	CBT-IA: 3-phase approach including behaviour modification to control compulsive Internet use, cognitive restructuring to identify, challenge, and modify cognitive distortions that lead to addictive use, and harm reduction techniques to address and treat co-morbid issues associated with the disorder Administered in 12 weekly sessions Sessions conducted between client and principle investigator Initial sessions gathered familial background, symptoms of the presenting problem, its onset, and severity CBT-IA addressed presenting symptoms related to computer use, specifically abstinence from problematic online applications and strategies to control use CBT-IA also focused on cognitive issues and harm reduction for underlying factors contributing to Internet abuse such as marital discord, job burnout, problems with co-workers, or academic troubles, depending on respective client Internet use routinely evaluated and treatment outcomes evaluated after 12 sessions and at 1, 3 and 6 mo follow-up	IAT[48]	Over 95% of clients managed symptoms at the end of the 12 wk period 78% sustained recovery six months following treatment CBT-IA ameliorated IA symptoms after 12 weekly sessions and consistently over 1, 3 and 6 mo after therapy
Yung *et al*[203]	To improve IAD involving Google Glass through residential treatment for alcohol use disorder	*n* = 1 (31-yr-old man who exhibited problematic use of Google Glass) Case report United States	Navys SARP All electronic devices and mobile computing devices customarily removed from patient during substance rehabilitation treatment 35-d residential treatment	N/A regarding SARP and measures, only about his reactions (*e.g.*, withdrawal, craving, *etc.*)	Following treatment, reduction in irritability, movements to temple to turn on device, and improvements in short-term memory and clarity of thought processes Patient continued to intermittently experience dreams as if looking through the device

Zhou *et al*[204]	To examine whether Internet addicted individuals share impulsivity and executive dysfunction with alcohol-dependent individuals	*n* = 66 22 IAD, 22 patients with AD, and 22 NC (NC consisting of citizens living in the city) China Experimental design	N/A	BIS-11 Go/no-go task Wisconsin Card Sorting Test (Beijing Ka Yip Wise Development Co., Ltd, computerized version VI) Digit span task Modified Diagnostic Questionnaire for Internet Addiction (YDQ)[66] Structured clinical interview (Chinese version) SADQ[205] Hamilton Depression Scale[206] Barratts Impulsivity Scale (BIS-11)[49]	Impulsiveness scores, false alarm rate, total response errors, perseverative errors, failure to maintain set of IAD and AD group significantly higher than that of NC group Hit rate, percentage of conceptual level responses, number of categories completed, forward scores, backwards scores of IAD and AD group significantly lower than that of NC group No differences in above variables between IAD group and AD group Internet addicted individuals share impulsivity and executive dysfunction with alcohol-dependent patients
Zhu *et al*[207]	To observe the effects of CT with EA in combination with PI on cognitive function and ERP, P300 and MMN in patients with IA	*n* = 120 patients in China with IA randomly divided into 3 groups: *n* = 39 EA group (*n* = 40, 27 male, mean age = 22.5, SD = 2.1 yr) *n* = 36 PI group (*n* = 25 male, mean age = 21.0, SD = 2.0 yr) *n* = 37 CT group (*n* = 40, 27 males, mean age = 22.5, SD = 2.3 yr)	Overall treatment period = 40 d EA applied at acupoints Baihui (GV20), Sishencong (EX-HN1), Hegu (LI4), Neiguan (PC6), Taichong (LR3), Sanyinjiao (SP6) and retained for 30 min once every other day PI with cognitive-behaviour mode every 4 d EA and PI used in CT group	Internet Addiction Test[208] Wechsler Memory Scale (WMS)[209] ERP observation[210] using MEB 9200-evoked detector Latency and amplitude of MMN and P300 recorded *via* EEG	Following treatment, IA decreased in all groups Decrease stronger in CT group relative to both other groups P300 latency depressed and amplitude raised n EA group MMN amplitude increased in CT group Short-term memory capacity and short-term memory span improved EA and PI improves cognitive function in IA *via* acceleration of stimuli discrimination and information processing on brain level

AD: Alcohol dependence; ADHD: Attention-deficit/hyperactivity disorder; AICA-C: Checklist for the assessment of internet and computer game addiction; AICA-S: Scale for the assessment of internet and computer game addiction; AUD: Alcohol use disorder; AUDIT-K: Korean version of alcohol use disorder identification test; BAI: Beck anxiety inventory; BDI: Beck depression inventory; BDI-Ⅱ: Beck depression inventory Ⅱ; BIS: Barratt impulsiveness scale; BIS-11: Barratt's impulsivity scale-11; BSD: Bipolar spectrum disorders; BSQ: Body sensations questionnaire; CAARS:S: Conners' adult ADHD rating scales self; CB: Compulsive buying; CBS: Compulsive buying scale; CBT: Cognitive behavioural therapy; CBT-IA: Cognitive-behavioural therapy for internet addiction; CDR: Centre for dependence rehabilitation; CDS-2: Cambridge depersonalization scale; C-FAI: Chinese family assessment instrument; CGI: Clinical global impression scale; CGI-I: Clinical global impressions-improvement scale; CIAS: Chen internet addiction scale; CIU: Compulsive internet use; CSEI: Coopersmith's self-esteem inventory; CT: Comprehensive therapy; DAPP: Dimensional assessment of personality pathology-short form; DC-IA-C: Diagnostic criteria of internet addiction for college students; DES: Dissociative experience scale; DSQ: Defense style questionnaire; EA: Electroacupuncture; EEG: Electroencephalogram; ED: Eating disorders; EDI-2: Eating disorder inventory 2; EDU: Education for internet use; EOP: Excessive online game play; EPI: Echo-planar image; ERP: Event-related potentials; GAD-7: Seven-item generalized anxiety disorder; GPIU: Generalised pathological internet use; GSE: General self-efficacy scale; HC: Healthy controls; IA: Internet addiction; IAD: Internet addiction disorder; IAG: Internet video game addicts; IAT: Internet addiction test; IC-IUD: Impulsive-compulsive internet usage disorder; IGD: Internet gaming disorder; IIP-D: Inventory of interpersonal problems; IRSQ: Interpersonal relationship styles questionnaire; ITC: Inferior temporal cortex; K-IAS: K-internet addiction scale; MDD: Major depressive disorder; MDQ: Mood disorder questionnaire; MET: Motivational enhancement therapy; MFGT: Mechanism of multi-family group therapy; MI: Motivational interviewing; MINI: Mini international neuropsychiatric interview; MMN: Mismatch negativity; MRI: Mental research institute; NC: Normal controls; NEO-FFI: NEO five factors inventory; NEO-PI-R: NEO personality inventory-revised; OCD: Obsessive compulsive disorder; OCS: Online cognitions scale; PA: Panic and agoraphobia scale; PCC: Posterior cingulate cortex; PD: Panic disorder; PG: Problematic gambling disorder; PHQ: Patient health questionnaire; PI: Psycho-intervention; POGP: Problematic online game play; QGU-B: Questionnaire on gaming urge-belief; ReHo: Regional homogeneity; SADQ: Severity of alcohol dependence questionnaire; SARP: Substance addiction recovery program; SCARED: Screen for child anxiety related emotional disorders; SCID: Structured clinical interview for DSM-Ⅳ; SCID-Ⅳ: Structured clinical interview for DSM-Ⅳ-patient version; SCL: Symptom checklist; SCL-90R: Symptom checklist 90-revised; SG: Survey group; SOC: Sense of coherence scale; STAXI-K: State-trait anger expression inventory; STG: Superior temporal gyrus; TG: Training group; YBOCS: Yale-brown obsessive compulsive severity scale; YIAS: Young's internet addiction scale; YIAS-K: Young's internet addiction scale, korean version; ZKPQ: Zuckermann-kuhlman personality questionnaire.

and escapism[164]. The latter was also used in another study[204].

A different approach was taken by Tao *et al*[163], who intended to develop diagnostic criteria for Internet Addiction Disorder (IAD) and to evaluate the validity of these criteria. Accordingly, in order to be diagnosed with IAD, patients had to fulfil the following criteria: The presence of preoccupation and withdrawal (combined with at least one of the following: Tolerance, lack of control, continued excessive use despite knowledge of negative effects/affects, loss of interests excluding the Internet, and Internet use to escape or relieve a dysphoric mood). In addition to this, clinically significant impairment had to be identified (*i.e.,* functional and psychosocial impairment), and the problematic behaviour had to last a minimum of three months, with at least six hours of non-essential Internet use a day. This study has been used as a basis for the APA's research classification of Internet Gaming Disorder in the DSM-5.

As this section demonstrates, a wide variety of measurements have been applied in order to ascertain Internet or Internet-use related addiction, sometimes involving an expert assessment by an experienced professional. As has been stated in previous research[6], no gold standard exists to measure Internet addiction with high sensitivity and specificity, which is exacerbated by the use of different cut-off points on the same measures across studies. To mitigate this diagnostic conundrum, a diagnosis of Internet addiction would significantly benefit from including a structured clinical interview administered by a trained professional[214], and this would help eliminating false positives and false negatives in the context of diagnosis.

Differential diagnoses/comorbidities

A number of studies investigated differential diagnoses and/or comorbidity of Internet addiction and other psychopathology. In terms of assessing potential comorbidities, the Structured Clinical Interview for DSM-Ⅳ mental disorders[64] was used by five studies[32,50,93,106,164]. Psychopathological symptomatology was also assessed using the Symptom-Checklist, SCL-90-R[125,191] and the Chinese version of the Mini International Neuropsychiatric Interview[115]. Personality disorders were identified by using the Dimensional Assessment of Personality Pathology-Short Form[58,59]. Other addiction-related assessments included alcohol and drug addiction measured with the DÉBA[76], the Alcohol Use Disorder Identification Test-Korean version[95], and the Severity of Alcohol Dependence Questionnaire[205], as well as shopping addiction, assessed *via* the Compulsive Buying Scale[54]. The presence of eating disorders was assessed using the Eating Disorder Inventory 2[52,53]. Mood disorders were assessed using the Hamilton Rating Scale for Depression[39], Beck's Depression Inventory[132], and the Mood Disorder Questionnaire[198]. Levels of anxiety were measured with the Hamilton Rating Scale for Anxiety[40], Beck's Anxiety Inventory[74], and the Generalized Anxiety Disorder scale (GAD-7)[127]. Symptoms of Attention Deficit Hyperactivity Disorder (ADHD) were investigated by means of Conners' Adult ADHD Rating Scales Self (CAARS:S)[42]. Finally, dissociation and depersonalisation were measured using the Dissociative Experiences Scale[34] and the Cambridge Depersonalization Scale[128].

The results of comorbidity and differential diagnosis analyses revealed the following. Of 50 adult outpatients self-referred for their Internet overuse, 14% presented with comorbid ADHD, 7% hypomania, 15% GAD, 15% social anxiety disorder, 7% dysthymia, 7% obsessive compulsive personality disorder, 14% borderline personality disorder, 7% avoidant personality disorder, and 2% binge eating disorder[32]. Higher frequencies of comorbid psychopathology were reported in a sample of 30 male patients with Internet gaming addiction[62], namely 40% antisocial personality traits, 56.7% affective disorders (30% major depression and 26.7% dysthymia), 26.7% other addictions (13.3% gambling, 10% alcohol, 10% marihuana, 6.7% nicotine and 3.3% cocaine addiction), and 16.7% antisocial disorders (13.3% ADHD, social phobia 10% and 3.3% dysmorphic corporal disorder). Generally smaller prevalence rates were reported in a sample of 57 Internet addiction treatment seekers in Canada[72]: 3.5% presented with comorbid depression and 7.5% with anxiety.

Half of a sample of 50 students with Internet addiction[79] presented with a comorbidity of another Axis Ⅰ disorder (10% with major depression, 5% with dysthymia and psychotic disorders, respectively). This finding was corroborated by another study of 290 male treatment seekers, half of whom met criteria for another psychiatric disorder[124]. In addition to this, of the former sample, 38% presented with a concurrent Axis Ⅱ personality disorder (22% with narcissistic, and 10% with borderline disorder, respectively)[79]. Significantly higher levels of depression and dissociation were furthermore found in a sample of 25 patients with Internet addiction as compared to a matched healthy control group[164]. Moreover, relative to a control group of male patients treated for alcohol addiction, 71 male patients with alcohol addiction and comorbid Internet addiction presented with higher levels of depression and obsessive-compulsive symptoms[188]. Furthermore, another study[197] including 368 Internet addiction treatment seekers showed that 30.9% met the diagnostic criteria for bipolar spectrum disorders, and this study also evidenced generally increased psychopathological symptomatology (including substance use disorders, affective and personality disorders). Finally, significant positive correlations were reported between compulsive buying and compulsive Internet use, as 11.7% of a sample of 60 female patients displaying patterns of compulsive buying also presented with addictive Internet use. This study reported no differences between individuals presenting with different types of eating disorders regarding compulsive Internet use[50].

Moreover, patients with Internet addiction and patients with pathological gambling received higher scores on depression, anxiety[26,27], and lower scores on global functioning relative to healthy controls, used impulsive coping strategies, and experienced more socio-emotional impairment. Additionally, patients with Internet addiction differed from patients with pathological gambling in that the former experienced higher mental and behavioural disengagement, which was found to be associated with interpersonal impairments[26].

Overall, the presence of comorbidities for Internet-use related addiction in the clinical context appears to be the norm rather than an exception. Individuals seeking treatment for their Internet overuse frequently present with mood and anxiety disorders, and other impulse-control and addictive disorders appear common. This indicates Internet addiction treatment may benefit from therapeutic approaches that combine evidence-based treatments for co-occurring disorders in order to increase treatment efficacy and acceptability for the patient.

Psychopharmacotherapy

In five studies, psychopharmacotherapy[20,22,24,28,46] for online addictions was used. Atmaca[28] reported the case of a 23-year-old male 4th year medical student who presented with the problems of problematic Internet use and anxiety. The patient was treated with a combination of selective serotonine reuptake inhibitors (SSRI) and antipsychotic medication. The antidepressant citalopram was administered at a dose of 20 mg/d and was increased to 40 mg/d within the period of a week, which was continued for six weeks. Subsequently, quetiapine (an atypical antipsychotic typically used for schizophrenia spectrum disorders) was added to the treatment, starting with a dose of 50 mg/d, which was increased to 200 mg/d within four days. The treatment resulted in decreased Internet addiction as measured with the Y-BOCS[30] modified for Internet use, decreased non-essential and essential Internet use, and improved control over Internet use. The improvements persisted until four-month follow up.

Bipeta *et al*[46] compared 34 control subjects with or without Internet addiction assessed *via* Young's Diagnostic Questionnaire[48] with patients with pure OCD with or without Internet addiction (mean age = 27 years, SD = 6.5 years). OCD patients were treated with standard pharmacological treatment for OCD (treatment as usual) for one year, received the benzodiazepine clonazepam (often used in the treatment of anxiety disorders), which was tapered off in three weeks, an SSRI or the tricyclic antidepressant clomipramine for 12 mo. The individuals with Internet addiction in the OCD group received the following doses of medication: Five patients received 150-200 mg fluvoxamine/d, four received 150-200 mg sertraline/d, one received 60 mg fluoxetine/d, and the final one received 200 mg clomipramine/d. In the OCD group that included individuals who were not addicted to using the Internet, the following doses of medication were administered: Eight patients received 150-300 mg fluvoxamine/d, five received 100-200 mg sertraline/d, eleven received 40-80 mg fluoxetine/d, and three received 150-200 mg clomipramine/d. Overall, the OCD treatment improved scores for both OCD and Internet addiction, while only two of the eleven OCD patients still fulfilled Internet addiction criteria after twelve months of treatment[46].

Dell'Osso *et al*[20] assessed the safety and efficacy of the antidepressant SSRI escitalopram (typically used for mood disorders) in 19 adult patients (12 men, mean age = 38.5, SD = 12.0 years) who presented with the problem of impulsive-compulsive Internet usage disorder assessed *via* the YBOCS[30], modified for Internet use. The trial consisted of a total of 19 wk, composed of a ten week treatment phase in which escitalopram was administered starting with 10 mg/d, and increased and maintained at 20 mg/d for 10 wk, and subsequent nine weeks of a randomised double-blind placebo controlled trial with or without administration of escitalopram at previous dosages. The treatment phase resulted in a significant decrease in Internet use. However, there were no differences in treatment effect between the treatment and placebo group following the second stage of the study. The authors also note that the group treated with escitalopram experienced negative side effects, including fatigue and sexual side effects, whereas side effects did not occur in the placebo group[20].

Han *et al*[26] used a controlled trial to test the effects of the antidepressant bupropion sustained release treatment (with a dose of 150 mg/d for the first week and 300 mg/d for five subsequent weeks) on the brain activity of eleven Internet video game addicts (mean age = 21.5, SD = 5.6 years), assessed *via* Young's Internet Addiction Scale[216]. The results indicated that the administered psychopharmacological treatment provided successful results for the video game addiction group, as it decreased craving, playing time, and cue-induced brain activity. These authors[22] also used the central nervous system stimulant concerta (methylphenidate commonly used for ADHD) in 62 video game playing children with ADHD (52 males, mean age = 9.3, SD = 2.2 years) who had not previously been given medication. Internet addiction was assessed using the Korean version of Young's Internet Addiction Scale[87]. The initial concerta dosage was 18 mg/d, with the maintenance dosage being individually adjusted based on the respective children's clinical symptoms and weight. Following treatment, Internet addiction and Internet use significantly decreased, as did ADHD symptoms and omission errors in a Visual Continuous Performance Test[22].

Taken together, the studies including psychopharmacological treatment for Internet addiction and/or gaming addiction showed positive effects in decreasing Internet addiction symptomatology and Internet/gaming use times. In the few studies conducted, antidepressant medication has been used most, suggesting mood

disorders may be comorbid with Internet use addiction. The research also indicated that if other (primary or secondary) disorders are present (specifically, OCD and ADHD), medication typically used to treat these disorders is also effective in reducing Internet addiction-related problems.

Psychological therapy

Ten studies[23,65,86,119,136,149,174,201-203] described some form of psychological therapy for treating Internet addiction. The majority of psychological therapies used an individual approach, which was applied to outpatients, apart from three studies that used group therapy approaches[23,65,119,136,149].

The most common approach used to treat Internet addiction was Cognitive Behavioural Therapy (CBT)[86,202]. This approach was usually individualised (apart from one study which used a group approach[65]). A further study used a combination of individualised and group therapy, namely Short-Term Treatment for Internet and Computer Game Addiction[192]. The typical CBT programme was administered for the duration of a few months, ranging from eight sessions[65] to 28 sessions, which included both group and individual sessions[192], and sessions lasted between one[86] and two hours[65]. The topics covered with patients in these sessions were: (1) identification of the Internet application associated with symptoms of addiction; (2) control issues (*e.g.,* examining the self, feelings, impulsivity, and the relation between the individual and the Internet to self-manage and self-restrain Internet use); (3) principles of healthy communication, namely interpersonal communication, such as between parent-children[65], and sharing success stories[86]; (4) Internet awareness (with regards to relationships established and developed through the Internet, and dealing with online content); (5) cessation techniques applied to the Internet (*e.g.,* recognizing the addictive behaviour and discontinuing it); and (6) additional elements (*e.g.,* college career planning, covering underlying factors contributing to Internet abuse, such as marital discord, job burnout, problems with co-workers, or academic problems). In general, CBT followed a number of stages, including team building or a probatory stage to review sessions or stabilization and relapse prevention. All sessions were run by therapists[119,192] or psychiatrists[86] who were supporting adults, apart from one case that involved children and adolescents[65].

The treatment outcomes were measured through scores on a number of psychometric scales covering excessive Internet use, including the Internet Overuse Self-Rating Scale[67,68], the Adolescent Pathological Internet Use Scale[120], the Internet Addiction Diagnostic Questionnaire[48], and the assessment of emotional, cognitive and behavioural symptoms. The following emotional skills and problems were measured in some studies. Anxiety was assessed using the Screen for Child Anxiety Related Emotional Disorders[71] and self-esteem was measured with Coopersmith's Self-Esteem Inventory[103]. Cognitive skills covered were diverse, and measures included the Online Cognition Scale (OCS)[80], and the Time Management Disposition Scale[69]. The behavioural characteristics related to Internet addiction primarily concerned the individual, but also included their peer and family relationships, and were measured using the Chinese version of the Strength and Difficulties Questionnaire[70], and the Parent-Child Communication Scale[122]. Only one study[86] did not make use of questionnaires because it was a neuropsychological and electrophysiological study conducted using an event-related potential approach, focused on cognitive function by detecting a P300 component. The results of this study indicated there was a deficit in cognitive functioning in Internet addicts, which is a finding that has also been observed in other addictive disorders[215].

Four of the included group therapy approaches (out of five studies) included Internet addicts and family groups treated simultaneously. These included (1) a CBT modality called "multimodal school-based group" (MSBG)[65]; (2) a "multi-family group therapy" (MFGT), which was used for treating Internet addiction for the first time[119]; (3) a traditional family therapy for a young adult addicted to using the Internet[136]; and (4) a "multi-level intervention model" that is usually applied to substance abuse, which included family counselling and peer support groups[149].

The psychotherapeutic MSBG approach was applied in a school setting and involved students, parents and teachers. The group of Internet addicts were students treated using classical CBT in a group ranging from six to ten participants. The students' parents were also administered cognitive behavioural training to recognize their children's Internet addiction (through children's feeling states, communication and solving-problem skills in the family, and through controlling the parents' own feelings and behaviours to manage their children's excessive Internet use). Teachers were provided psychoeducation, which was delivered by means of workshops in didactic teaching, analysis and discussion, with the purpose of recognising and treating Internet addiction in students, and of supporting their parents.

MFGT is a new psychotherapy approach for adolescent Internet addicts[119]. This intervention provides therapeutic groups for both adults (parents) and adolescents (Internet addicts), and the aim is to provide peer support, allowing transferential reactions, engagement with the treatment and promoting family cohesion. The main goal of this form of psychotherapy is to reduce Internet addiction whilst improving parent-adolescent communication and closeness, and to fulfil the family members' psychological needs, rather than these needs being fulfilled by Internet use. Altogether, six active sessions were used, with a subsequent three-month follow-up to target potential relapse and discuss new issues and solutions to maintain the effectiveness of the intervention. Each of the sessions lasted for two hours and included five parts: a warm-up exercise, feedback on homework from the last session, a main structured

activity, a brief summary and the family assignment. The topics treated per session were: Understanding a family with the problem of excessive Internet use (session 1), parent-adolescent communication skills training (session 2), parent-adolescent communication practices related to the problem (sessions 2 and 3), parent-adolescent relationship building skills training (session 4), associations between psychological needs and Internet use, how to satisfy the unfulfilled need in the family relationships (session 5), and setting up healthy expectations for the family system (session 6).

The classical family therapy approach used in one study[136] was based on Bowen's[216] family system theory, which focused on the distinction of the self-inside from the self within the family constellation, and was based on an extensive analysis of family-of-origin problems and communication patterns. The treatment was focused on current interactions and changes in behaviour in the family system[217] to modify the family's communication method by changing behaviours that maintain problematic Internet use, and coping with Internet overuse related problems. The therapy focused on an undesirable online behaviour and replacing it with a healthy behaviour, which would simultaneously induce a change in the family relationships. The intervention lasted three months and included 15 sessions. It treated emotional problems to enhance control over Internet use, and included functional and emotional expression to solve interpersonal relationship problems associated with Internet addiction.

The multi-level intervention model included an individual-based counselling approach with motivational interviewing (MI), complementary techniques, and traditional family-based counselling[149]. It consisted of six phases, lasting between 15 and 19 mo. The phases included (1) emphasising controlled and healthy Internet use; (2) promoting understanding of the change process through different stages from pre-contemplation to relapse, (3) using the MI model[218] for Internet addiction; (4) adopting a family perspective by using a systemic approach; (5) applying a multi-level counselling model including the patient, his/her family and his/her peers; and (6) using individual and group therapy to facilitate the intervention.

The only group approach that did not include a family intervention was the R/T group counselling programme, which specifically addressed Internet addiction[23]. It consisted of ten group sessions (two per week) within the period of one month, which varied in length between one and 1.5 h. Accredited specialists provided this intervention for university students. The content included an introduction to the therapy goal, teaching, activities, homework assignment and sharing. Each session furthermore included four sections: The purpose, materials (*e.g.,* blank paper, topic-oriented games, posters, videos), strategies (*e.g.,* discussion topics, homework assignments) and session evaluation for both the individual and their family, in order to assess whether the aim of the sessions had been achieved.

Overall, the psychological studies which included a control group to compare the effect of the interventions achieved varying results, impeding a general analysis of psychotherapy impact. Du *et al*[65] did not find significant differences between experimental and control groups in the post-test measure of Internet overuse, although the intervention group improved their time management (efficacy and time control) and other skills (emotional symptoms, conduct problems, hyperactivity, peer relationships and prosocial behaviours) significantly, and this was maintained until a six-month follow-up. Other comparative findings included a longer P300 component duration in Internet addicts treated by CBT compared to healthy controls[86]. However, the amplitudes were similar in both groups. Moreover, although Internet addiction symptoms were reduced after treatment in the experimental group[149], this was not the case for the group's scores on beliefs and behaviours related to Internet use and psychological well-being, and there was only a small improvement in parental monitoring and functioning following treatment.

Only two studies (out of four experimental studies) showed a clear effectiveness of psychological therapy, and both of these used a group approach. Kim[23] used a quasi-experimental design and an intervention with a group psychotherapy approach, and found a significant reduction in Internet addiction and significantly higher self-esteem in the experimental group compared to the control group. Liu *et al*[119] found that their MFGT approach was effective in three aspects. It resulted in a significant reduction of time spent online (reduced by half in comparison to the controls), a decrease in the Internet addiction measure, and, from the parents' perspective, more satisfaction regarding their child's online behaviours. Moreover, the most important factor to reduce Internet addiction in this study was found to be the parent-adolescent relationship.

Combined therapy

Six studies used combined therapy to treat Internet addiction, consisting of some form of psychological treatment in combination with one of the following: Other psychological therapies[138,180], pharmacotherapy[21,25,139] or electroacupuncture therapy[221].

CBT was the most frequently applied psychological therapy to treat Internet addiction. Subsequently, add-ons to the CBT approach included in the identified studies will be elaborated on. Motivational Enhancement Therapy (MET) was developed by Poddar *et al*[138] and was tested in the context of treating IGD. This MET-CBT approach consisted of a series of stages: (1) a contemplation stage (*i.e.,* initial sessions of rapport building, a detailed interview and case formulation); (2) a preparation stage (*i.e.,* sessions delivered in an empathetic atmosphere to emphasise psychoeducation, including managing physiological and emotional arousal through relaxation techniques, and a cost-benefit analysis of game addiction); and (3) a contract stage with the patient, a parent and the therapist (*i.e.,* behaviour

modification of gaming, reducing time spent online and promoting healthy activities). By applying these stages, a reduction of IGD and online gaming was achieved, and school performance was improved.

Another case study[139] combined CBT with psychopharmacotherapy [*i.e.,* administering clonazepam (a benzodiazepine typically used to treat anxiety disorders) and sertraline (an SSRI antidepressant)] to treat Internet addiction. The intervention lasted for three months, and consisted of the following. The CBT approach aimed to support self-recognition and modify and restructure feelings and dysfunctional cognitions related to Internet use, with the goal to prevent relapse. CBT was administered for ten weekly sessions to teach the patient to handle her anxiety and other symptoms related to her Internet use (in this case panic and obsessive symptomatology, which was comorbid to her Internet addiction). Clonazepam (0.5 mg) and sertraline (50 mg) were also administrated once daily. The applied treatment proved effective for reducing both anxiety and Internet addiction.

A new treatment approach to treat Internet addiction combined CBT and MI with an on-the-job Lifestyle Training programme[180]. Treatment was delivered by qualified therapists who were supervised by a senior therapist for both main psychological therapies. The treatment consisted of eliciting and strengthening the motivation to change, choosing a treatment goal, gaining self-control, preventing relapse, and coping skills training. Ten outpatient sessions of 45 min were used, and seven of these took place within a period of 2.5 mo. The remaining sessions were optional and were administered as a follow-up within 3 mo. Each of the sessions had a fixed format: (1) introduction; (2) evaluation of current status; (3) discussing homework; (4) explaining the theme of the day; (5) practicing a skill; (6) receiving homework; and (7) closing the session. This study was the only study that provided three perspectives for data collection: The patients', the therapists' and the researchers' perspectives. This intervention, which is commonly used for other addictive disorders, was found to work well for Internet addiction as it reduced Internet use, increased social contacts, provided a daily structure, and encouraged alternative uses of free time and positive beliefs.

Moreover, CBT was most frequently used in combination with a psychopharmacological treatment, such as administering bupropion. The reason to select this medication is because a proportion of patients with major depressive disorder (MDD) are also excessive online gamers, and this drug has been previously evaluated as potential treatment for MDD and other drug-addictions. Recently, its effectiveness has been tested and confirmed experimentally[21,25]. Han and Renshaw[21] tested this combined treatment in Chinese male adolescent and adult patients with mood disorders and online gaming addiction, and treated them with bupropion sustained release (from 150 mg/d until 300 mg/d during 8 wk) and a psychological intervention (*i.e.,* education for Internet use). The treatment resulted in significantly decreased depression and Internet addiction levels, and time spent playing online games compared with the control group. At follow-up (*i.e.,* four weeks post treatment), the reduction in gaming hours and level of Internet addiction was maintained, while the depression recurred.

Similarly, Kim *et al*[25] tested the effectiveness of CBT in an active treatment group *vs* a control group who did not receive CBT in Korean male adolescent patients with MMD and online gaming addiction. Both groups were treated using the same levels of bupropion. Following treatment, Internet addiction was significantly reduced in the CBT group and other measures showed improvement (*e.g.,* anxiety and life satisfaction), while depression severity did not change. These findings were maintained at follow-up. Therefore, the combination of psychotherapy with bupropion is effective in MDD patients with online gaming addiction in the long term only for online gaming addiction, and the time spent using online games. Both studies with bupropion were managed by psychiatrists, and one[25] used a multidisciplinary treatment team including a psychiatrist, nurse, psychologist, and social worker.

One study used clonazepam (0.5 mg/d) and sertraline (50 mg/d) combined with CBT to treat Internet addiction[139]. This study reported the case of a young Brazilian woman with Internet addiction and comorbid psychiatric disorders (*i.e.,* panic and OCDs). During the treatment period of ten weeks, both drugs were administered daily whilst CBT was provided once a week, and focused on teaching the patient how to handle anxiety and Internet use through breathing training with diaphragmatic exercises, education about both disorders' symptoms and about Internet use (*e.g.,* time management, triggers of problematic Internet use, changing habits, cognitive restructuring, exposure and response prevention, promotion of social support, alternative activities, and promotion of functional Internet use). This combined treatment was effective for all conditions treated.

Zhu *et al*[207] combined a psychological intervention (*i.e.,* CBT with sessions every four days for a total treatment period of 40 d) with electroacupuncture in 120 patients presenting with Internet addiction in China. They used three groups: 40 participants in the electroacupuncture group, 36 participants in the psychological intervention group, and 37 individuals participated in the comprehensive therapy group combining both treatment ingredients. Electroacupuncture was applied at acupoints Baihui (GV20), Sishencong (EX-HN1), Hegu (LI4), Neiguan (PC6), Taichong (LR3), and Sanyinjiao (SP6), and retained for 30 min once every other day. Overall, treatment was effective in all groups as Internet addiction symptomatology was successfully decreased, whereas this effect was significantly stronger in the combined therapy group relative to the other groups. The authors furthermore note that the combined treatment improved cognitive function in Internet

addiction by means of accelerating stimuli discrimination and information processing on the level of the brain.

Combined therapies have shown effective results for treating Internet addiction, including both post-treatment and follow-up measures. The use of electroacupuncture in combination with a psychological intervention improved treatment success for Internet addiction more than providing cognitive-behavioural treatment only, suggesting the novel therapy electroacupuncture may be beneficial in the treatment of Internet addiction. It is suggested to replicate this study to verify the positive results.

Conversely, given the results found by the included studies, psychopharmacotherapy does not always appear to be as efficacious for psychological problems, such as major depression, as it is for Internet and gaming addiction. This is an interesting finding, because it seems that Internet addiction is usually accompanied by other psychological disorders. Therefore, combining therapies may be a good option for some clients, and should be managed by interdisciplinary teams with structured mid-term interventions.

DISCUSSION

This systematic literature review has sought to provide an overview of the currently available clinical research on Internet addiction and problematic Internet use using a holistic perspective. Clinical studies concerning Internet addiction, problematic Internet use and excessive online gaming have been included to offer a comprehensive insight into the relevant research to date. A total of 46 empirical clinical studies were identified, which focused on treatment seeker characteristics and different types of therapy provisions. Treatments included psychopharmacotherapy, psychological therapy, and combined treatment. Each of these will be discussed subsequently.

In terms of treatment seeker characteristics, the included studies indicated that the published research ranged from case studies to including patients treated for problematic Internet use in both inpatient and outpatient settings across 13 countries and four continents. It is worth noting that a number of studies indicated that comorbidities appear to be the norm, rather than an exception for individuals who present with the problem of Internet addiction or problematic Internet use. Comorbid mood and anxiety disorders appear to be particularly common. A link between mood disorders and Internet addiction has been suggested in previous research, including both adolescent[88,210-227] and adult samples[228-233]. A possible explanation for this strong and frequent link may be the fact that as Internet use increases, online activities take up gradually more time in the lives of Internet users. This reduces the time available to participate in alternative enjoyable pastime activities and to engage with real-life family and friendship circles, which may lead to increased loneliness and stress[234]. Alternatively, Internet use and gaming may serve as a method to escape real-life problems, effectively resulting in avoidance coping, which may exacerbate stress and negative feelings, and lead to negative consequences, including addiction and depression[235].

Moreover, a number of earlier studies have shown that anxiety disorders and anxiety-related symptoms, including social phobia, phobic anxiety, and OCD co-occur with Internet addiction in adolescents[88,236-238] and adults[230,238]. Previous research including Internet addiction treatment experts from six countries indicated that a large percentage of individuals presenting with Internet addiction at both in-patient and out-patient treatment facilities suffer from comorbid anxiety disorders, most commonly social anxiety and social phobia[11]. This may be explained through the mechanism of compensation, suggesting individuals who have difficulties engaging and bonding with their peers in real life may instead use the Internet for social interaction, as the online space removes the embodied (and potentially anxiety-provoking) elements from the interaction. These elements include the individual's outward appearance and the exclusion of (often feared) face-to-face contact in favour of virtual (and often text-based) interaction. This may facilitate social interaction by increased likelihood of self-disclosure[239], online disinhibition[240], and hyperpersonal communication, characterised by the increased speed of developing social bonds and intimacy online[241].

The research presented indicated that comorbidities complicate treatment. This literature review has shown that comorbidities are very common in the context of Internet addiction, emphasising the necessity to investigate the extent to which Internet addiction can be considered a primary or a secondary disorder (*i.e.,* secondary to some other psychopathology). Researchers have suggested that given the presence of comorbidity, it is questionable whether Internet addiction deserves an individual diagnosis, as this may lead to other (primary) disorders being underdiagnosed. This may lead to problems regarding efficient treatment choices on behalf of the mental healthcare professionals given that efficacious treatments exist for the more prevalent disorders, such as anxiety and mood disorders[242], whereas the evidence base for Internet addiction treatment is still rather limited in comparison. However, research has also indicated that some symptoms of Internet addiction appear as stand-alone symptoms and can be differentiated from other psychopathology, providing empirical evidence for the discriminant validity and specificity of the Internet addiction construct[243]. If comorbidity is present in individuals presenting with Internet addiction or problematic Internet use, clinicians need to target both problems in treatment as research has indicated that individuals with comorbid psychopathology (specifically co-occurring Axis I mental disorders) present with more clinical problems[79].

In terms of psychopharmacotherapy, the five studies included in this systematic literature review showed that SSRIs (*i.e.,* citalopram, clomipramine, fluvoxamine, sertraline, fluoxetine, escitalopram), norepinephrine-dopamine reuptake inhibitors (NDRI; *i.e.,* buproprion), benzodiazepines (*i.e.,* clonazepam), antipsychotic medication (*i.e.,* quetiapine), and methylphenidate *(i.e.,* concerta) were used to treat Internet addiction and Internet-use related problems. Overall, in the included studies, the use of psychopharmacological treatment to alleviate Internet and gaming addiction symptomatology and time spent online appeared successful, suggesting that Internet addiction is an indication for the use of the administered medications[20,22,24,28,46].

The diverse range of administered medication corresponds with the diverse range of presenting problems of the samples included. For instance, concerta is a drug which is efficacious in treating ADHD and therefore commonly used in ADHD treatment[244] as it has been shown to improve inhibition, motivation and memory by increasing dopamine and norephinephrine concentrations in the brain[245]. Moreover, given the relatively high prevalence of both mood and anxiety disorders with comorbid Internet addiction as described above, it is not surprising that antidepressant medications and benzodiazepines are frequently used in the pharmacological treatment of Internet addiction. SSRIs are the method of choice for mood and anxiety disorders and related symptoms[246], and benzodiazepines have anti-anxiety and relaxing properties[247]. Despite their off-label status in countries including the United Kingdom and Australia, NDRIs are often prescribed for depression-related symptoms and disorders[248]. In sum, the studied psychopharmacological treatments for Internet addiction proved efficacious in decreasing both Internet addiction symptoms as well as symptoms of other psychopathologies for which the specific medications have been licensed. Even so, clinicians need to assess the costs and benefits of the medication they are prescribing for treating Internet addiction as some side effects may impact treatment acceptability and treatment adherence in patients.

Regarding psychological therapy for Internet addiction and problematic Internet use, ten studies were identified, most of which used a group therapy framework to support clients. Group therapy has a number of advantages over individual therapy. According to the American Psychological Association[249], the benefits of group therapy include establishing a support network of individuals who experience similar problems and are faced with similar difficulties. Other group members' stories may put the patients' own problems into perspective. Moreover, group therapy may create a safe environment in which the sensitive topic of Internet-use related addiction can be discussed openly. Group therapy has the benefit of offering the possibility to learn from others and consequently improve coping skills as individuals differ in their ways they face the world and deal with their lives. These benefits explain why group therapy frameworks are popular psychological therapies for Internet addiction and Internet use-related problems.

The addition of the family network into therapy sessions as evidenced in studies on multimodal school based groups[65], MFGT[119], family therapy[136], and a multi-level intervention model[149] appears particularly fruitful for young patients, as families are important social groups supporting the young patients' development. Families teach values, offer emotional attachment, model appropriate behaviours, and discourage high-risk behaviours[250]. The efficacy of group-based and systemic therapy for adolescents with problems of substance use and addiction has been long established[251], and suggests that therapeutic frameworks derived from family-based therapies for these disorders may be similarly efficacious in the treatment of Internet addiction and problematic Internet use. The included studies have verified this contention, and therefore clinicians are advised to incorporate families in the psychological treatment of young patients (including adolescents and young adults).

The most commonly applied therapy form was CBT or some variation thereof (*e.g.,* CBT-IA)[202], which has frequently been used in an individual format. The primary goal of CBT is to change maladaptive cognitions and behaviours associated with Internet use, and this therapy form is in line with Davis'[252] cognitive-behavioural model of pathological Internet use. The model suggests cognitive factors are particularly important in the development and maintenance of Internet addiction. In the included studies, cognitive measures indicated that CBT is efficacious in reducing cognitive impairment associated with Internet addiction[86]. However, Winkler *et al*[17] examined the efficacy of different treatments for Internet addiction in a meta-analysis which included 13 studies, and their results showed that CBT did not perform significantly better than other psychological treatments, although CBT appears to be the most popular approach for treating Internet addiction.

Finally, a number of studies have simultaneously included different forms of therapy, namely psychological treatment supplemented with other types of psychological therapy[138,180], pharmacotherapy[21,25,139] or electroacupuncture therapy[219]. Taken together, all of the combined therapies were efficacious in treating Internet use-related problems, whereas the benefits for comorbid psychopathology (*e.g.,* depression) were limited. This suggests that in cases where comorbidity is present and psychopharmacological treatment is administered, the clinician and researcher need to carefully monitor the patient's progress, adjust the dosage of the medication and/or change the medication administered to achieve the best possible results for the patient. Moreover, as the new treatment modality of electroacupuncture outperformed psychological interventions, it is suggested that researchers replicate these positive results to ensure they hold across other samples.

A number of limitations need to be highlighted in

the included studies. Only a few studies (*e.g.*,[20,21,23,24,26,27,46,65,78,93,109,119,133,143,188,204]) included a control group, making it difficult to ascertain whether the positive effects of treatment on Internet addiction symptom and related problem reduction were due to the administered treatment, or to non-specific factors of treatment [*i.e.*, the placebo effect (the improvement of symptoms with no treatment)], which can be due to natural history and statistical regression to the mean, among other factors[253]. Moreover, a lack of intention-to-treat analysis in the reported studies might have caused bias in the results due to treatment non-compliance, changes from the initial treatment protocol, or leaving out data from individuals who dropped out of the study before or during the course of treatment[254].

For future research, the need to utilise validated and reliable measures of Internet addiction and/or problematic Internet use needs to be stressed. Currently, the diagnostic and research landscape appears particularly broad, and diagnostic criteria used to identify the potential disorder are not globally agreed upon. Researchers are recommended to collaborate to establish a consensus regarding diagnostic criteria and measures in order to improve the reliability across studies and to develop effective and efficient treatment approaches for treatment seekers. This will furthermore contribute to providing an incentive for public policy and healthcare providers to offer funding for those who need professional help. Ultimately, research and clinical initiatives need to focus on providing the best possible care for individuals who experience significant impairment and distress as a consequence of their Internet use.

COMMENTS

Background

Over the last 15 years, the number of Internet users has increased by 1000%, and at the same time, research on addictive Internet use has proliferated. Internet addiction has not yet been understood very well, and research on its etiology and natural history is still in its infancy. In 2013, the American Psychiatric Association included Internet Gaming Disorder in the appendix of the updated version of the Diagnostic and Statistical Manual for Mental Disorders as condition that requires further research prior to official inclusion in the main manual, with important repercussions for research and treatment.

Research frontiers

To date, reviews have focused on clinical and treatment studies of Internet addiction and Internet Gaming Disorder. This arguably limits the analysis to a specific diagnosis of a potential disorder that has not yet been officially recognised in the Western world, rather than a comprehensive and inclusive investigation of Internet-use related addictions (including problematic Internet use) more generally.

Innovations and breakthroughs

The aim of this literature review is to provide a comprehensive overview of clinical studies on the clinical picture of Internet-use related addictions from a holistic perspective.

Applications

Researchers are recommended to collaborate to establish a consensus regarding diagnostic criteria and measures in order to improve the reliability across studies and to develop effective and efficient treatment approaches for treatment seekers. This will furthermore contribute to providing an incentive for public policy and healthcare providers to offer funding for those who need professional help. Ultimately, research and clinical initiatives need to focus on providing the best possible care for individuals who experience significant impairment and distress as a consequence of their Internet use.

Terminology

Internet addiction is a condition that requires further research prior to official inclusion in the diagnostic manuals, with important repercussions for research and treatment. To date, reviews have focused on clinical and treatment studies of Internet addiction and Internet Gaming Disorder. This arguably limits the analysis to a specific diagnosis of a potential disorder that has not yet been officially recognised in the Western world, rather than a comprehensive and inclusive investigation of Internet-use related addictions (including problematic Internet use) more generally.

Peer-review

In this systematic review, the authors have presented a thorough and critical analysis of clinical research on Internet addiction related studies.

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SYSTEMATIC REVIEWS

White matter alterations in anorexia nervosa: A systematic review of diffusion tensor imaging studies

Beatriz Martin Monzon, Phillipa Hay, Nasim Foroughi, Stephen Touyz

Beatriz Martin Monzon, Phillipa Hay, Nasim Foroughi, Centre for Health Research, School of Medicine, Western Sydney University, Sydney 2751, Australia

Stephen Touyz, School of Psychology, Faculty of Science, University of Sydney, Sydney 2006, Australia

Author contributions: Martin Monzon B and Hay P designed the study purpose; Martin Monzon B and Foroughi N collected the studies; Martin Monzon B analysed the data and wrote the paper; all authors contributed with the revisions and approved the final manuscript.

Conflict-of-interest statement: Professor Touyz has received fees for serving as consultant to Shire Pharmaceuticals advisory board member. Professor Hay receives Honoraria from PLOS Medicine. In the past Professor Hay has received reimbursement of expenses for speaking at medical meetings and attending symposia from Astra-Zeneca, Solvay Pharmaceuticals, Bristol-Myers Squibb, and Pfizer Pharmaceuticals, and for educational training for family doctors fromBristol-Myers Squibb, Pfizer Pharmaceuticals and Lundbeck and has been funded by Jansen-Cilag to attend educational symposia (none in the past 10 years). Professor Touyz and Professor Hay receive royalties from Hogrefe Publications, McGraw Hill Pubs and honoraria from Biomed Central. Beatriz Martin Monzon receives research funding and support from the Western Sydney University in the form of an International Scholarship.

Data sharing statement: Technical appendix, statistical code, and dataset are available from the corresponding author at p.hay@westernsydney.edu.au. As this was a systematic review of published data there were no participants to be approached for informed consent for data sharing. No additional data are available.

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Correspondence to: Phillipa Hay, Foundation Chair of Mental Health, Centre for Health Research, School of Medicine, Western Sydney University, Locked Bag 1797, Penrith, Sydney 2751, Australia. p.hay@westernsydney.edu.au
Telephone: +61-02-46203838
Fax: +61-02-46203891

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Abstract

AIM: To identify findings concerning white matter (WM) fibre microstructural alterations in anorexia nervosa (AN).

METHODS: A systematic electronic search was undertaken in several databases up to April 2015. The search strategy aimed to locate all studies published in English or Spanish that included participants with AN and which investigated WM using diffusion tensor imaging (DTI). Trials were assessed for quality assessment according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses checklist and a published quality index guideline.

RESULTS: A total of 6 studies met the inclusion criteria, four of people in the acute state of the illness, one included both recovered and unwell participants, and one included people who had recovered. Participants were female with ages ranging from 14 to 29 years. All studies but one measured a range of psychopathological features. Fractional anisotropy and mean diffusivity were the main DTI correlates reported. Alterations were reported in a range of WM structures of the limbic

system, most often of the fornix and cingulum as well as the fronto-occipital fibre tracts, *i.e.*, regions associated with anxiety, body image and cognitive function. Subtle abnormalities also appeared to persist after recovery.

CONCLUSION: This diversity likely reflects the symptom complexity of AN. However, there were few studies, they applied different methodologies, and all were cross-sectional.

Key words: Eating disorder; Diffusion tensor imaging; Weight/shape overvaluation; Food anxiety

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Core tip: The present systematic review identifies the latest research on white matter (WM) brain alterations in anorexia nervosa (AN). The WM architecture has been poorly understood due to its structure forming deep parts of the brain. It transmits information between cortical and subcortical structures. New advances in imaging methods with diffusion tensor imaging, allow its characterization and integrity analysis. Alterations in areas of fornix, cingulum, corpus callosum, cerebellum, superior longitudinal fasciculus and thalamus have been found in AN. They could be related to symptoms like anxiety, body image perception, reward processing and cognitive abilities.

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INTRODUCTION

Anorexia nervosa (AN) is a serious illness and has the highest standardized mortality ratio of all psychiatric disorders[1]. According to the DSM-5 criteria, a person in the sick state is underweight for their for age and height, fears gaining weight or becoming fat, and persists in behaviours to avoid weight gain, and weight/shape overvaluation or body image disturbance[2]. In young people it is the third commonest chronic illness and has a mortality rate 12 times higher than the death rate from all causes in young women[3]. In addition to acute effects of self-starvation there may be severe long term medical and psychological sequalae[4].

AN is multidetermined, and as well as psychological and environmental aetiologies, there is increasing evidence for biological underpinnings of its pathophysiology[5]. These include genetic, neuroendocrinologic and metabolic pathways. Furthermore, studies of the brain structure in this mental disorder have identified structural abnormalities. Severely underweight individuals with AN have altered brain function that is considered more profound than found in any other psychiatric disorder[6]. First computed tomography and then functional magnetic resonance imaging and other neuroimaging techniques have been used to study brain alterations in AN[7]. (FMRI measures the brain activity by detecting associated changes in blood flow, on the premise that cerebral blood flow reflects neuronal activation[8]).

The mechanisms addressing brain alterations remains unclear, but changes in brain grey matter (GM) and white matter (WM) might underlie altered brain function and behaviour[9]. Both fibres are part of the central nervous system (CNS) which co-ordinates basic activities of the human body. The GM is related to processing and cognition whilst the WM co-ordinates different brain regions. The first studies found differences in the GM of AN compared to healthy people and other eating disorders. However, the peculiar architecture of WM and the limited availability of imaging methods to study the WM delayed its investigation. Now, new advances in imaging procedures such as diffusion tensor imaging (DTI) allow the study of the structure of WM and its implication in the pathology of AN.

Volumetric brain imaging studies have been used to study GM and WM volume abnormalities in underweight adolescent and adult people with AN[10]. Several have reported loss of global and regional GM and often WM volume. After 2 or 3 d of dehydration these volumes are significantly lower, whereas hyperhydration is associated with higher GM and WM volumes[11].

Functionally connected brain regions utilising WM axons are considered to modulate higher order human behaviours, cognitions and emotions[12]. Investigating WM functionality thus may elucidate the cortical networks and circuitries that underpin the clinical features of AN. Kazlouski *et al*[13] (2011), were the first to use DTI to identify microstructural WM alterations in adults with AN. They found decreased fractional anisotropy (FA) in the cingulum, fronto-occipital and fimbria-fornix WM tracts.

The normal maturation and reorganization of WM during adolescence and young adult years is thought likely to be disturbed and explain in part the onset of AN[14]. WM fibres such injured WM fibres may not recover in those with enduring illness and years of starvation[15]. Simultaneously, other studies associate nutritional status with quickly occurring GM and WM changes[11].

A variety of studies have previously used the DTI technology showing reduced FA in a multitude of regions across mood, psychotic, anxiety and substance use psychiatric disorders[16]. In AN, the limited research has just began to identify functionally related brain areas structures[13]. Therefore, added to the difficulty to identify AN prior to illness onset, it remains unclear if the structural brain abnormalities are a prerequisite or

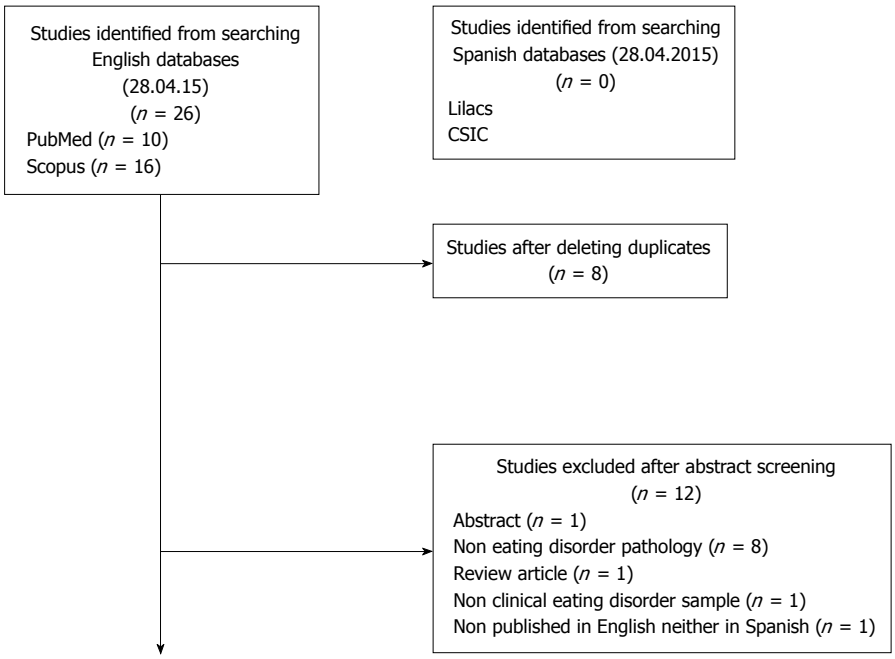

Figure 1 Selection process of the retrieved studies.

consequence of the development of AN[5].

Rationale for the current review

Findings are very heterogeneous with respect to WM abnormalities regarding to brain region and behaviour-relationship. Hence, the aim of the current systematic review is to critically examine and synthesise the existing research that has investigated WM microstructural alterations in individuals with AN using DTI. Specifically, the functions of implicated brain areas and relationships with psychopathological measures, and implications for understanding the pathophysiology of AN will be described.

MATERIALS AND METHODS

Search strategy

The search strategy was designed to identify all studies of subjects with eating disorders in which microstructure of brain WM brain fibres was analysed through DTI. The literature search was conducted in November 17th 2014 in SCOPUS and Pubmed electronic databases. The following search terms were used: "DTI" AND ("AN" OR "eating disorders"). All fields were included in order to expand the quest. Trials were incorporated if they were published in English and peer-reviewed.

Secondly, we included an additional search in the Spanish language. In December 17th 2014, we introduced the following terms on Lilacs and CSIC electronic databases: "imagen de tensor de difusión" Y ("anorexia nerviosa" O "trastornos de alimentación"). The findings were non-existent in in both of these databases.

In order to update the search, on April 28th 2015 we conducted a third data search on SCOPUS, PubMed, Lilacs and CSIC electronic databases. We used the terms previously described.

A total of 26 papers were retrieved, from which 8 duplicates were removed. The studies were screened to determine the suitability according to this review by two authors independently. Data extraction and quality assessment were done by Beatriz Martin Monzon, checked for agreement by Nasim Foroughi and disagreements were reviewed by Phillipa Hay. According to the inclusion criteria, 7 papers were excluded based on the abstract, leaving a total of 6 articles for full review. A detailed map of the search strategy stage is shown in Figure 1.

Selection of the studies

In order to prevent selection bias, the following inclusion criteria were decided on prior to the review. The studies should be: (1) published in a peer-reviewed journal; (2) available either in English or Spanish; (3) participants were over 14 years of age and under 65 years of age; (4) the experimental group should be composed of participants who met the diagnostic criteria for current or lifetime AN according to DSM-5 or DSM-IV criteria; (5) the control group should be composed of healthy participants; and (6) correlates of brain WM fibre were assessed using DTI measures.

Papers were excluded if they were dissertations, review articles or abstracts. No restrictions were placed on year of publication, gender of participants and body mass index (BMI).

Quality assessment

This systematic review was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses guidelines[17].

The final retrieved articles were assessed using the

Table 1 Baseline characteristics of the included studies (all cross-sectional in design)

Ref.	AN subtype	ED subtype	Study purpose	Method DTI	Assessment
Kazlouski *et al*[13]	Current AN restricting and purging subtype	AN purging and restricting subtype	Test of the brain WM integrity alteration and its relation to heightened anxiety in AN	GE 3 Tesla whole-body MRI scanner, maximum gradient amplitude of 40 Mt/M and maximum slew rate of 150 T/m per second, 8-channel phased-array head coil	SCID-CV EDI-3 TCI STAI BDI
Frank *et al*[49]	Current AN restricting and purging subtype	AN	Analysis of GM and WM in a sample of adolescents in comparison to adults	Signa 3T Scanner, axial, 3-dimensional, T-1 weighted magnetization-prepared rapid acquisition gradient echo	C-DISC EDI-3 TCI STAI CDI SPSRQ 9 point Likert scale sweetness-pleasantness of 1 molar sucrose
Nagahara *et al*[7]	Current AN, NS subtype	AN	Exploration of WM abnormalities in AN patients compared to HC	Whole body 3 Tesla MR system with an eight-channel phased-array head coil	SCID-CV EDI-2 BDI-II
Via *et al*[53]	Current AN restricting subtype	AN	Assessment of WM microstructure in a clinical sample of AN patients	GE Signa Excite scanner at 1.5 T equipped with an 8-channel phased-array head coil	SCID-CV SCID-I/NP GHQ-28 EDI-2 HAM-D HAM-A
Frieling *et al*[5]	Current AN with NS subtype and recovered women from AN	AN and recovered women from AN	Assessment of the microstructural integrity of WM pathways through DTI in women with AN	3 T Siemens-scanner, with a gradient field strength up to 45 MT/m	None
Yau *et al*[39]	Recovered women from restricting type AN	Recovered women from restricting type AN	Examination of the microstructural alterations of WM integrity in recovered AN women	3.0 Tesla Discovery MR750 scanner with an 8-channel phase-array head coil	SCID-CV MINI TCI STAI MPS

AN: Anorexia nervosa; DTI: Diffusion tensor imaging; GM: Gray matter; HC: Healthy controls; NS: Non-specified; WM: White matter.

Quality Index from Downs and Black[18]. The "checklist for measuring study quality" is a tool for assessing the quality of randomised and non-randomised trials, which provides a profile of their methodological strengths and weaknesses. It has reported high internal (KR-20: 0.89) and external consistency (KR-20: 0.54), tested using the Kuder-Richardson formula 20[19].

RESULTS

Description of studies

The 6 studies selected are indicative of the advances in the DTI technique for investigating WM fibre in AN patients. Table 1 presents the main characteristics of the included studies and Table 2 the characteristics of their participant samples. Three of the studies were nested in United States, one in Japan, one in Germany and another in Spain.

All the studies included a group of AN people and a control or healthy group. From this total, 2 studies also included recovered AN women as one of the groups of participants investigated. As inclusion criteria, it was mandatory to be diagnosed as AN according to the DSM-IV criteria and that the BMI was measured. All of them were comprised of women with mean ages ranging from 14 to 29 years old.

All 6 papers used a cross-sectional design. Three studies used a Tesla scanner, 2 a Signa scanner and one a Siemens model scanner for the diffusion tensor templates. Five papers used self-report questionnaires to assess psychopathological characteristics and one did not report use of assessment instruments.

Table 3 presents the main outcomes of each study and author's interpretation of the findings. Following the DTI procedures, the brain areas selected by the authors as possibly shown abnormalities are the fornix (and its connection to the fimbria tract), the cingulum, the thalamus (several nuclei), the superior longitudinal fasciculus, the corona radiata, the cerebellum and the fronto-occipital fibre tracts. Their main functions are emotion processing, body perception, reward processing, anxiety and cognitive style.

Quality assessment

Overall, the studies met most of the quality criteria considered for this review (Table 4). A maximum score of 8 points based in a yes/no/partially scale response was developed to grade quality. The score range was from 4 to 7 points, with mean of 5.75 and SD of 1.57.

Specifically, all the papers described the hypothesis or main study objective. The main outcomes to be investigated were clearly described in the Introduction

Table 2 Characteristics of the participant samples in the reported studies

Ref.	AN subtype	Total size	Sample characteristics *n* Gender Mean ± SD: Age/years BMI Duration of illness/years ED group (AN)	Recovered AN	Healthy group
Kazlouski *et al*[13]	Current AN restricting and purging subtype	*n* = 33	*n* = 16 (10 resAN, 6 purgAN) Female 23.9 ± 7 SD 16.5 ± 1 SD 7.5 ± 8 SD	-	*n* = 17 Female 25.1 ±4 SD 21.5 ±1 SD NA
Frank *et al*[49]	Current AN restricting and purging subtype	*n* = 41	*n* = 19 (17 resAN, 2 purgAN) NS 15.4 ± 1.4 SD 16.2 ± 1.1 SD NS	-	*n* =22 Female 14.8 ± 1.8 SD 21.3 ± 1.9 SD NA
Nagahara *et al*[7]	Current AN, NS subtype	*n* = 35	*n* = 17 Female 23.8 ± 6.68 SD 13.6 ± 1.3 SD 4.93 ± 4.9 SD	-	*n* = 18 Female 26.2 ± 5.6 SD 19.9 ± 2.0 SD NA
Via *et al*[53]	Current AN restricting subtype	*n* = 38	*n* = 19 Female 28.37 ± 9.55 SD 17.03 ± 1.09 SD 6.52 ± 6.03 SD	-	*n* = 19 Female 28.63 ± 8.58 SD 21.09 ± 1.80 SD NA
Frieling *et al*[5]	Current AN with NS subtype and recovered women from AN	*n* = 41	*n* = 12 Female 26.8 ± 6.94 SD 15.18 ± 1.39 SD NS	*n* = 9 Female 27.44 ± 5.3 SD 19.31 ± 1.39 SD NS	*n* = 20 Female 24.80 ± 2.6 SD 19.60 ± 0.94 SD NA
Yau *et al*[39]	Recovered women from restricting type AN	*n* = 22	-	*n* = 12 Female 28.7 ± 7.9 SD 21.2 ± 1.5 SD 5.6 ± 5.2	*n* = 10 Female 26.7 ± 5.4 SD 22.0 ± 1.1. SD NA

AN: Anorexia nervosa; recAN: Recovered anorexia nervosa; purgAN: Purgative subtype anorexia nervosa; resAN: Restrictive subtype anorexia nervosa; BMI: Body mass index; NA: Non-applicable; NS: Non-specified.

or Method section. The participant characteristics were clearly described, often being displayed in a chart or table. The intervention of interest wasn't applicable to the present systematic review, since none of the retrieved studies developed an interventional design. The main weakness of the studies was the description of the confounders' distribution. This was clearly described in one, partially in two and not provided in three studies. Adjustment for confounding was reported in three studies only. Also, most studies did not include another mental health disorder such as depression in participants in a control group. This was clearly described in one study, partially in two studies and not provided in three studies. The main study findings were clearly described in all the papers. All but one study, which didn't provide enough information, selected appropriate statistical tests for each research study measurement.

Measures used

DTI measures reported in the six included papers: Water diffusion direction along the WM tracts was measured with FA and MD was used as an indicator of white matter integrity, a scalar measure reflecting the magnitude of diffusion. Mean diffusivity (MD) or in other term apparent diffusion coefficient provides information about the average diffusion-freedom water molecules have in the brain tissue, and correlates with local cell breakdown[20-22].

Psychopathological measures: To confirm the diagnoses of AN, five of the 6 collected studies used the Structured Clinical Interview for DSM-IV Axis I Disorders. Specifically, four studies used the clinician version[23] and one of them included the non-patient edition for the sample group[24] and the General Health Questionnaire[25]. One of the studies chose the Computerized Diagnostic Interview for Children[26] and one The Mini-International Neuropsychiatric Interview[27].

In general, the majority of the studies used the same assessment instruments for general eating disorders symptoms, depression as well as anxiety. The presence of symptoms and psychological features

Table 3 Main diffusion tensor imaging outcomes and author interpretation

Ref.	AN subtype	Outcomes: DTI measures and areas in AN	Author interpretation
Kazlouski *et al*[13]	Current AN restricting and purging subtype	FA: Lower in the bilateral fimbria-fornix, fronto-occipital and cingulum fiber tracts	Anxiety is predicted by the fimbria-fornix FA value. Thus, reduced WM integrity could provide a mechanism for heightened anxiety
Frank *et al*[49]	Current AN restricting and purging subtype	FA: Lower in fornix, cingulum and corpus callosum (corona radiata and forceps mayor)	Abnormal fornix integrity could lead to altered feedback between limbic and higher-order brain structures. The corpus callosum could be implicated in taste processing
Nagahara *et al*[7]	Current AN, NS subtype	FA: Lower value in the left cerebellum MD: Higher value in the fornix	WM abnormalities in the fornix and the cerebellum may be neural substrates of the pathophysiology of AN. The fornix is one of the important components of the Papez circuit, which links the limbic system with other brain structures. The correlation of WM alteration with physical severity, including BMI and duration of illness may indicate that WM alteration is more relevant with regard to physical severity rather than psychological severity
Via *et al*[53]	Current AN restricting subtype	FA: Lower in the parietal part of the left SLF and the fornix. MD: Higher in the SLF and the fornix. They also reported significantly increased MD in the fornix, accompanied by decreased FA and increased RD and AD	The left SLF seem relevant to body image distortion as well as other cognitive processes like the called weak central coherence. The fornix is a key structure involved in the regulation of body-energy balance and processing of reward responses
Frieling *et al*[5]	Current AN with NS subtype and recovered women from AN	FA (AN and recAN): Lower in the posterior thalamic radiation bilaterally (which includes the of optic radiation) and the left mediodorsal thalamus	The posterior thalamic radiation fibres project to areas involved in the processing of the body image, whose alteration could explain the AN body image distortion. The left mediodorsal thalamic nucleus is connected to regions contributing to impairments in cognitive domains, especially set/shifting ability, executive control, habit learning and reward processing
Yau *et al*[39]	Recovered women from restricting type AN	FA (recAN): Insignificant alteration MD: Lower in frontal, parietal and cingulum	Lower MD was associated with harm avoidance, suggesting a possible underlying trait associated with AN. Localization of disturbances in frontal-parietal and cingulum WM suggests that these pathways, which are important for cognitive control, may be susceptible to core AN pathology. Malnutrition seems to have potentially lasting effects on WM integrity and degree of recovery

AD: Axial diffusivity; AN: Anorexia nervosa patients; HC: Healthy controls; FA: Fractional anisotropy; MD: Mean diffusivity; recAN: Recovered from AN; SLF: Superior longitudinal fasciculus; WM: White matter; RD: Radial diffusivity.

Table 4 Quality assessment of the retrieved studies

Ref.	Q1 Hypothesis clearly described	Q2 Main outcomes clearly described in the introduction or methods section	Q3 Participants characteristics clearly described	Q5 Distributions of confounders in each group of subjects clearly described	Q6 Main study findings clearly described	Q15 Study measurement blind	Q18 Appropriateness of the statistical tests	Q25 Adjustment for confounding
Kazlouski *et al*[13]	Yes	Yes	Yes	No	Yes	U	U	No
Frank *et al*[49]	Yes	Yes	Yes	Partially	Yes	U	Yes	Yes
Nagahara *et al*[7]	Yes	Yes	Yes	No	Yes	U	Yes	No
Via *et al*[53]	Yes	Yes	Yes	Yes	Yes	U	Yes	Yes
Frieling *et al*[5]	Yes	Yes	Yes	Yes	Yes	U	Yes	Yes
Yau *et al*[39]	Yes	Yes	Yes	No	Yes	U	Yes	No

Q4 regarding to interventions of interest clearly described was not applicable to this review since any of the studies undertook an interventional design. Yes scored as 1, No scored as 0, Partially scored as 0.5. U: Unable to determine.

typically presented in AN was assessed through the eating disorders inventory-3[28] in two studies whilst its second version[29] was used in other 2. Three studies complemented it with the temperament and character inventory[30]. Mood was assessed with the beck depression inventory first edition (BD Ⅰ)[31] and its second edition (BD Ⅰ-Ⅱ)[32] in two studies. Three studies used the state-trait anxiety inventory[33] to measure anxiety levels. Other questionnaires applied the hamilton rating scale for depression[34] and the hamilton rating scale for anxiety[35,36], the children's depression inventory[36], the revised sensitivity to reward and

punishment questionnaire[37] and the multidimensional perfectionism scale[38]. In addition, in one study a molar sucrose and a control solution for sweetness and pleasantness were rated on a 9-point Likert scale.

DISCUSSION

The neurobiology of AN is still poorly understood. This systematic review reports research that investigated WM fibre tracts using advances in DTI procedures. Sample selection criteria, type of scanner and psychopathological measures used, as well as DTI correlates with brain functions connections were documented. Six studies were included and WM fibre changes were found in tracts relevant to emotional brain circuits as well as to neurocognitive brain structures.

Summary of findings

Four of the six included studies performed the WM fibre measurement in patients at the sick state, one included recovered AN patients and another one studied the possible WM alterations in recovered and as well AN acute patients. As a control measure, some studies included the indicator of low BMI in the healthy sample to make the groups comparable. The criteria for recovery differed in the two studies that included such participants. Specifically, in the study of Frieling *et al*[5], the participants didn't meet criteria for a diagnosis of AN at the time of inclusion, and had a BMI above 18 km/m^2. In contrast, Yau *et al*[39] defined recovery based on symptoms of no food restriction, no compulsive exercise and not engaging in any other ED behaviours as well as sustaining a stable weight (± 3 kg and 90% to 120% of mean body weight) with regular menstrual cycles.

Four of the studies found either a low FA value or a high MD correlate in the fornix. This formation is an important WM structure that originates from the hippocampus as the fimbria-fornix tract and projects superior-anteriorly toward the midline, forming the body of the fornix[40]. It is a part of the Papez Circuit, an element of the limbic system involved in the regulation of emotions by higher order frontal cortical brain regions. It is linked to the hypothalamus, the amygdala as well as to prefrontal areas like the ventral striatum, the orbitofrontal cortex and the cingulate cortex[41]. The fornix is a key connection involved in the regulation of body-energy balance and processing of reward responses. It has been associated with trait anxiety and harm avoidance and responds to leptin (feeding inhibiting hormone)[42]. The last studies suggest that fornix alterations in AN could be involved in the characteristic of food restriction, disrupted meal patterns, altered food reward processing and difficulties making behaviour changes[43]. Previous studies in rodents have identified those altered patterns[44].

Another well reported brain structure in three of the retrieved studies is the cingulum. It is composed of a WM association fibres bound from the cingulate gyrus to the entorhinal cortex in the brain. It is part of the limbic system which connects frontal lobe regions with posterior structures as the temporal lobe and hippocampus. They comprise a network that integrates the necessary behaviours for executive function and emotion recognition[45]. Alteration in this structure could be related to altered emotion identification and processing in AN, as well as disturbances in cognitive control[46].

At the same time, two studies found reduced FA in fronto-occipital association fibres. These tracts connect frontal with occipital and posterior parietal and temporal lobes. They integrate auditory and visual association cortices and may have a role in the spatial awareness and neglect, as well as emotion recognition and expression[47]. The fronto-occipital WM abnormality could be related to altered body perception shown in AN. It has been associated with abnormal recognition of emotions in others and emotion regulation within themselves[48].

Frieling *et al*[5] (2012) identified bilateral reductions of FA maps in the posterior thalamic radiation and the left mediodorsal thalamus. Alterations of the posterior thalamic radiations are of special interest because they are connected with the occipital, temporal and parietal lobes which are all involved with the cortical processing of body image perception. In contrast, the left mediodorsal thalamus is linked to brain structures implied in cognitive domains related to the shifting ability, executive control, habit learning and reward processing.

Alterations of WM fibre were seen as well in adolescent AN samples. Frank *et al*[49] (2013) registered a low FA in the corpus callosum. This area facilitates communication between left and right sided brain structures and has been implicated in taste processing. Parent *et al*[50] (1996) found an alteration in the corona radiate and forceps major, a group of bundles that connect the cerebral cortex with the basal ganglia and spinal cord. Both structures are related to taste, whose significance could be related to altered taste and processing in AN[51].

There are several brain structures that integrate a functional network connecting the prefrontal and parietal areas which may be implicated in the body image distortion found in AN. The superior longitudinal fasciculus is the major association WM tract connecting these regions[52]. Accordingly, Via *et al*[53] (2014) reported significant FA decreases in the parietal areas of the superior longitudinal fasciculus, which sets the basis for the representation of the body self-image[54]. Proprioception, size and spatial judgment, visual imagery and the integration of visual information are properties of body image. Apart from that, the authors suggested the possible implication of the superior longitudinal fasciculus in other cognitive processes such as the "weak central coherence" reported in people with AN. This cognitive style is characterized by an enhanced attention to local details at the expense of global processing and is found in several disorders[55]. These abnormalities are thought to depend on alterations of

long-range connections between prefrontal and parieto-occipital areas, which could be also implicated in AN[56].

One of the studies[39], reported altered WM patterns in parietal areas of AN subjects. The authors found reduced diffusivity in fronto-parietal areas that was associated with cognitive control to be related to increased post-error slowing during response inhibition[57]. These results are consistent with increased cognitive control and error perseveration[58]. Thus, a low FA value may be associated with typical AN traits such as enhanced cognitive control and clinical perfectionism[6].

Moreover, it has been seen that abnormalities in the left cerebellum exist in AN patients. Nagahara *et al*[7] (2014) found a low FA value in the lateral zone of this structure. It projects to the dentate nucleus which has connections with the ventral anterior nucleus and ventral lateral nucleus of the thalamus. Added to the functions that we have specified previously, the thalamus facilitates coordinated movements and has a direct bidirectional connection with regions involved in the regulation of food intake. Specifically, the connections with several hypothalamic nuclei (the lateral hypothalamic areas, the dorsomedial hypothalamic nucleus and the ventromedial hypothalamic nucleus) could prompt abnormal food intake behaviour in AN[59]. Furthermore, the cerebellum also relates social, empathic and emotional function, including fear response[60]. Altogether, the alterations of these functions may underline the pathophysiology of AN[61].

In sum, the pathophysiology of AN is still unknown but the limbic system is undoubtedly implicated as many of the DTI studies found alterations in this network. The connections with the prefrontal areas might explain the dysfunction which appears in the cognitive style and emotion procession. Two main theories are considered in understanding these neurocognitive mechanisms in AN. The alterations in WM fibre could reflect predisposing personality traits and neurocognitive style. On the other hand, the effects of undernutrition could cause these abnormalities in WM.

Limitations and future research

Assessment instruments varied between the studies based on the analysis method. Most of the studies selected a combined method of assessment including cognitive assessment *via* questionnaires and MRI scanning for analysis of the WM fibre. Although some of the questionnaires were commonly used in most of the studies, the diverse range of used questionaries is problematic for comparing between studies. There was also diversity in the type of scanner used for imaging, the patient's position in the scanner, and the selected regions of interest.

Previous studies have analysed specified DTI measures, including FA and MD. The axial diffusion coefficient has also been used in some of the previous studies. Future studies should consider investigating the relationship between the DTI measures and indirect measures. The exposed FA and MD are broad measures that could be driven by a number of factors (*e.g.*, axonal ordering, density, degree of myelination). For this reason, axial diffusivity and radial diffusivity (RD) are thought to index more specific aspects of WM pathology, being more sensitive to changes in integrity and myelination, respectively. The combined quantification of direct and indirect measurements is recommended to better characterize any putative changes in WM structure.

All the studies used a cross-sectional design and there is a need for longitudinal studies to examine the WM alterations over time to determine if changes found in the recovered state are a persistent "scare" of illness effects such as starvation, or are innate to the disorder. Furthermore, future research should consider more comprehensive examination of myelination changes in people with AN. Changes in RD have been observed after different periods of cognitive treatment or meditation. This may reflect a brain plastic response that could provide new insights for understanding recovery in AN.

Finally, this review was limited in that only four databases were used and two of which were English language databases. However these two were the most relevant databases to the topic, and one, Scopus, is a composite of papers from many sources including several other databases such as MEDLINE and EMBASE. Insufficient funding for translations also precluded sourcing literature published in languages excepting those which the authors were fluent in.

In conclusion, the results of our review suggest that WM changes exist in brain fibres of people with AN. The research also showed that the changes appear in the brain during the sickness stage and the recovery stage in patients with AN. The main brain alterations seem to involve tracts of the limbic system, as the fornix. A prompt treatment plan for the sickness and recovery stages seems crucial for minimising the progression of brain impairments. However, there were few studies and problems of inconsistent assessment methodologies and a need for replication of findings and longitudinal study design.

COMMENTS

Background

There is evidence for biological underpinnings in anorexia nervosa (AN) that argue in favour of structural brain abnormalities. Previous research performed with Voxel Based Morphometry has found reduced grey matter (GM) volumes in people with AN compared to healthy controls. White matter (WM) is the second major component of the central nervous system (CNS) and it coordinates communication between different brain regions.

Research frontiers

Very little research has been performed on WM in AN participants due to limited technologies able to investigate its particular structure. New advances with diffusion tensor imaging (DTI) however have identified alterations in several areas of the brain WM providing new insights into the structure and related

function of WM in people with AN.

Innovations and breakthroughs

This review synthesises all the studies to date (April 2015) using DTI to assess the WM integrity in AN. Alterations in a range of WM structures of the limbic system were identified in regions associated with anxiety, body image and cognitive function. Subtle abnormalities also appeared to persist after recovery but no studies were prospective.

Applications

This systematic review synthetises high quality research evidence related to WM brain alterations in AN. It provides researchers with the latest knowledge available and directions for future research.

Terminology

WM: Component of the CNS that distributes information between different brain areas; GM: Component of the CNS related to processing and cognition activities; DTI: Magnetic resonance imaging method that allows the mapping of the diffusion of molecules in the brain, *in vivo* and non-invasively. It is able to delineate fibre tracts within the WM; Fractional anisotropy (FA): A value that describes the degree of anisotropy of a diffusion process. It is thought to reflect myelination of WM; Mean diffusivity: An opposed value to FA which reflects the isotropy of the diffusion.

Peer-review

The present systematic review aims to identify findings concerning WM fibre microstructural alterations in AN. The authors find that WM fibre is altered in diverse areas of brain, the diversity possibly reflecting the symptom complexity of the AN.

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