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Room 903, Building D, Ocean International Center,
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EDITORIAL

Mavridis' atrophy in Parkinson's disease-five years later: Future perspectives

Ioannis N Mavridis, Efstratios-Stylianos Pyrgelis

Ioannis N Mavridis, Department of Neurosurgery, "K.A.T.-N.R.C." General Hospital of Attica, 14561 Athens, Greece
Efstratios-Stylianos Pyrgelis, Department of Neurology, "K.A.T.-N.R.C." General Hospital of Attica, 14561Athens, Greece
Author contributions: Mavridis IN and Pyrgelis ES reviewed the literature and wrote this paper.
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Correspondence to: Ioannis N Mavridis, MD, PhD, Department of Neurosurgery, "K.A.T.-N.R.C." General Hospital of Attica, Nikis str. 2, Kifissia, 14561 Athens, Greece. pap-van@otenet.gr
Telephone: +30-697-8327199
Fax: +30-210-2833600
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Abstract

Mavridis' atrophy (MA) is called the human nucleus accumbens (NA) atrophy in Parkinson's disease (PD). MA begins in early-stage PD patients and is correlated with psychiatric symptoms that occur in PD, mainly apathy and impulsive behavior. It is also associated with cognitive PD symptoms. Purpose of this editorial was to discuss the future perspectives of MA as a pathological and imaging finding. MA is obviously part of the degeneration of the dopaminergic nigrostriatal system that occurs in PD and this also explains the fact that MA precedes clinical phenotype. But does the human NA follow the same pattern of degeneration? It would be quite interesting to have a post-mortem pathological study focused on the NA of parkinsonic individuals. Further questions that remain to be answered are whether all parkinsonics suffer MA and whether this phenomenon is also associated with motor PD symptoms. MA as an imaging finding could be a risk factor for the expression and/or severity of specific PD symptoms. It has therefore to be tested whether the presence of MA is related, for example, with the expression and/or severity of motor PD symptoms and whether the severity of MA affects the severity of specific psychiatric symptoms (apathy, compulsive behavior) of parkinsonic individuals. Such clinical studies, that could provide answers to these vital questions, can be easily preformed given the high frequency of PD in modern populations. Future research efforts are mandatory to enrich our knowledge of MA, namely its underlying mechanisms, its pathological features and its clinical consequences.

Key words: Parkinson's disease; Mavridis' atrophy; Nucleus accumbens; Neuroimaging; Neuropathology; Substantia nigra

Core tip: Mavridis' atrophy (MA) is the nucleus accumbens atrophy in Parkinson's disease (PD). MA begins in early-stage PD patients and is correlated with psychiatric and cognitive PD symptoms. MA is obviously part of the dopaminergic nigrostriatal degeneration that occurs in PD. It would be interesting to have a post-mortem pathological study focused on the nucleus accumbens of parkinsonic individuals. MA as an imaging finding could be a risk factor for the expression and/or

severity of specific symptoms. Thus it has to be tested whether the presence of MA is related, for example, with the expression and/or severity of motor PD symptoms.

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MAVRIDIS' ATROPHY

Parkinson's disease (PD), a common neurodegenerative disorder, is predominantly a disorder of basal ganglia, which are a group of nuclei situated deep and centrally at the base of forebrain and their main components are the striatum (caudate nucleus, putamen, nucleus accumbens), the globus pallidus, the substantia nigra (SN) and subthalamic nucleus[1-3].

The human nucleus accumbens (NA) is a major part of the ventral striatum. Connected to the limbic and extrapyramidal motor system, it has a modulating function in the amygdala-basal ganglia-prefrontal cortex circuit and is considered as the neural interface between motivation and action (limbic-motor interface). It is a modulator of the reward circuits (major pleasure center) of the human brain and thus involved in several cognitive, emotional and psychomotor functions, as well as in some of the commonest neurological and psychiatric disorders, including PD[3].

Mavridis' atrophy (MA), discovered five years ago as an imaging finding, is called the human NA atrophy in PD[4-10]. Several new data regarding MA were published during the last few years. More specifically, MA, confirmed by recent clinical studies, begins in early-stage PD patients and is correlated with psychiatric symptoms that occur in PD, mainly apathy and impulsive behavior. The MA phenomenon is also associated with cognitive PD symptoms[10]. Purpose of this editorial was to discuss the future perspectives of MA as a pathological and imaging finding.

FUTURE PERSPECTIVES

Parkinson's pathology and Mavridis' atrophy

The recognized neuropathological findings in PD are: (1) The degeneration which leads to cell death of the pigmented neurons in the pars compacta region of the SN that produce the neurotransmitter dopamine. The loss of dopaminergic neurons occurs most prominently in the ventral lateral SN. Approximately 70% of these neurons are lost before the motor signs of PD emerge[11]; and (2) The presence of Lewy bodies (cytoplasmic inclusions) in perikarya and Lewy neuritis in the neurons which result in premature cell death of the affected neurons. Their prevalence increases with age, but they are specific to PD and are found in some cases of synucleinopathies and other disorders[11,12].

PD belongs to a group of neurodegenerative disorders called a-synucleinopathies which are characterized by the intracellular presence of a neuronal protein called α-synuclein, the major component of Lewy bodies (LBs). Primary α-synucleinopathies include PD, dementia with Lewy bodies and multiple system atrophy, where the α-synuclein deposition occurs in oligodendrocytes rather than neurons. While all α-synucleinopathies are characterized by α-synuclein aggregates with similar posttranslational modifications and lipid associations, the cell type involved, their location and their association with other protein depositions vary substantially[13]. A-synuclein aggregation in the form of LBs has been also reported in neurodegenerative diseases that are not synucleinopathies, specifically in Alzheimer's disease, Pick's disease and in corticobasal degeneration[14,15]. In the last two cases the LB are detected within the cytoplasm of the characteristic balloon neurons[15].

PD is morphologically featured not only by the degeneration of the dopaminergic nigrostriatal system, responsible for the motor deficits, but also by multifocal involvement of the central, peripheral and autonomic nervous system and other organs associated with widespread occurrence of Lewy bodies and dystrophic Lewy neurites. This results from deposition of abnormal α-synuclein, the major component of Lewy bodies and the main protein marker of PD and of other synucleinopathies[16,17].

Regarding the pathological changes that characterize MA, it seems obvious this is part of the degeneration of the dopaminergic nigrostriatal system and this also explains the fact that MA precedes clinical phenotype. It should be noted here that the SN is one of the very few areas of the human brain which are connected to the NA with both afferent and efferent fibers[3,6]. But does the human NA follow the same pattern of degeneration? Do Lewy bodies present in the NA neurons of parkinsonic patients? Probably yes. But is this presence related with the process of MA? It would be quite interesting to have a post-mortem pathological study focused on the NA of parkinsonic individuals. Further questions that remain to be answered are whether all parkinsonics suffer MA and whether MA is also associated with motor PD symptoms.

Parkinson's imaging and MA

Until recently, conventional magnetic resonance imaging was usually negative in PD or showed nonspecific findings. Recent developments in structural MRI, such as relaxometry, magnetization transfer and neuromelanin imaging, have demonstrated improved contrast and enabled more accurate visualization of deep brain nuclei, in particular the SN, and cortex[18,19]. Meanwhile, diffusion imaging has provided useful biomarkers of SN degeneration, showing reduced anisotropy and anatomical connectivity with the

striatum and thalamus[18,20]. The most well-developed MRI markers in PD include diffusion imaging and iron load using T2/T2* relaxometry techniques. Other biomarkers such as susceptibility-weighted imaging for iron load, magnetization transfer and ultra-high-field MRI have shown great potential[19]. These advances in structural imaging are complemented by findings of magnetic resonance spectroscopy on brain metabolism and resting-state functional MRI on functional connectivity[18]. Using resting-state functional MRI, for example, it has been found that the presence of apathy, one of the most common neuropsychiatric symptoms in PD affecting 23%-70% of patients, is associated with functional connectivity reductions in frontostriatal circuits, predominating in the left hemisphere and mainly involving its limbic components[21]. It has also to be mentioned that brain perfusion can be assessed using non-contrast-agent techniques such as arterial spin labeling and that spectroscopy gives access to metabolites' concentrations[19].

Regarding the MA as an MRI finding in PD, it is time to evaluate the usefulness of its observation in clinical practice. It could be a risk factor for the expression and/or severity of specific PD symptoms. Thus it has to be tested whether the presence of MA is related, for example, with the expression and/or severity of motor PD symptoms and whether the severity of MA affects the severity of specific psychiatric symptoms (apathy, compulsive behavior) of parkinsonic individuals. Such clinical studies, that could provide answers to these vital questions, can be easily preformed given the simplicity of their methodology and the high frequency of PD in modern populations. Current MRI viewing software can be very helpful in identifying and even quantifying NA shrinkage. Finally, it would be also interesting to study the NA functional imaging of parkinsonic patients.

Finally, hypothesizing which type of motor symptoms could be linked to MA, we should mention hypokinetic and akinetic symptoms, because these have been suggested as some of the possible clinical consequences of MA[9]. But could the early identification of MA using neuroimaging techniques serve to prevent the disability associated with PD? Given that we are currently just seeing the "peak" of the "iceberg" called "MA"[10], we cannot be sure yet. It is highly probable that it is expected to help in setting diagnosis in a quite early or even preclinical stage of the disease, since MA is already present in early-stage PD[8]. And this should force us to focus on prevention and treatment efforts in such stages that could, if not prevent, at least delay the progression of PD-related disability. And this could be a significant step forward.

CONCLUSION

In conclusion, MA is an interesting new finding in PD, confirmed by recent clinical data. It is for sure that further research efforts are mandatory to enrich our knowledge of MA, namely its underlying mechanisms, its pathological features and its clinical consequences. We believe for example that MA is associated with motor PD symptoms, an interesting hypothesis that remains to be confirmed by clinical studies. So, future research should be directed to the clinical usefulness of MA and its relative applications.

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REVIEW

Targeting remyelination treatment for multiple sclerosis

Maheen Nadeem, Lindsay Sklover, Jacob A Sloane

Maheen Nadeem, Lindsay Sklover, Jacob A Sloane, Multiple Sclerosis Center, Beth Israel Deaconess Medical Center, Boston, MA 02115, United States

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Correspondence to: Jacob A Sloane, Director, Multiple Sclerosis Center, Beth Israel Deaconess Medical Center, 330 Brookline Ave, Ks212, Boston, MA 02115, United States. jsloane@bidmc.harvard.edu
Telephone: +1-617-6673742
Fax: +1-617-6671869
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Abstract

Since disability in multiple sclerosis (MS) is a product of neurodegeneration and deficient remyelination, the ability to enhance neuroregeneration and myelin regeneration in MS is an enticing goal for MS drug development. In particular, remyelination treatments could promote return of neurological function and also prevent further axonal loss and neurodegeneration in MS due to trophic effects of myelin. The study of remyelination has advanced dramatically in the last several years such that a number of pathways inhibiting remyelination have been discovered, including those involving LINGO-1, Notch-1, hyaluronan, retinoid X receptor, and wnt/ß-catenin. Other approaches such as high throughput drug screening for remyelination drugs have caught fire, with identification of dozens of known drugs with oligodendrocyte maturation stimulatory effects. Several drugs identified through screens and other mechanisms are in the process of being further evaluated for remyelination in MS and MS models. We discuss the potential molecular targets and the variety of mechanisms towards drug identification and development in remyelination for MS.

Key words: Multiple sclerosis; Myelin; Remyelination; Oligodendrocyte; Repurposing; Treatment

Core tip: Over the last several years numerous remyelination pathways important to multiple sclerosis (MS) have been identified, including those of LINGO-1, hyaluronan, Notch-1, retinoid X receptor receptor, and wnt/ß-catenin. Newer discoveries include the pathways involving Chemokine (C-X-C Motif) ligand 12/C-X-C chemokine receptor type 4 and G protein-coupled receptor 17, and the involvement of Endothelin-1 in the Notch pathway. High-throughput screens have identified multiple antimuscarinic drugs with good remyelination. Also identified by screens, clemastine, with similar antimuscarinic but also antihistamine effects, may be useful in remyelination in MS. Drug repurposing, through screens or more serendipitously, has found that many drugs could enhance remyelination, including benztropine, clemastine, quetiapine, fasudil, olesoxime, and ibudilast, among others.

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INTRODUCTION

Multiple sclerosis (MS) is a debilitating disease of the central nervous system (CNS) that affects nearly 2.5

million people worldwide and is characterized by the predominant presentation in young women compared to men[1]. It manifests through an autoimmune inflammatory response which damages the protective myelin coating on axons, leading to disrupted neurological functioning[1]. Continued relapses can lead to irreversible damage within the CNS, driving clinical deterioration[2]. The MS spectrum ranges from 50%-85% of people initially diagnosed with MS beginning with the relapsing-remitting form of MS (RRMS); after approximately 10-20 years, most patients with RRMS transition to secondary progressive MS which is characterized by irreversible neurological decline[3]. MS takes a toll both physically and mentally within the patients, leading to a disease that requires management entailing high financial costs to both patients and health care systems.

The basis of neurological disability arises from neurodegeneration, defects in myelination, and continued inflammation[1]. Neurodegeneration develops over time in MS patients as a consequence of autoimmune inflammation and incomplete remyelination. Since myelin is attacked by autoimmune inflammation, damage can be primarily demyelination with axonal preservation, and this alone can cause neurological dysfunction unless repaired.

No drugs are currently in use to help prevent neurodegeneration or poor remyelination in MS. Drugs currently approved by the Food and Drug Administration (FDA) for MS treatment are immunomodulators/immunosuppressors that prevent or limit inflammation from occurring but do not directly repair damage already incurred on axons and myelin. Understanding that neurodegeneration and poor remyelination is the primary source of disability in MS, there is avoid in MS treatments aimed at remyelination in MS. This paper outlines the search for remyelination enhancing agents through drug repurposing and high throughput screens aimed at isolating an agent that removes the roadblocks to remyelination in the MS CNS.

NATURAL REMYELINATION IN MS

Remyelination can be a natural reparative mechanism in MS. Remyelination appears to require activation of oligodendrocyte progenitor cells (OPCs), recruitment of OPCs to lesions, and cellular differentiation of OPCS to become myelin-producing oligodendrocytes[4]. Remyelination occurs in the brains of some MS patients though not as complete as to allow for full return of normal function. In one post-mortem study, MS white matter lesions were 47% remyelinated on average and 22% of them were found to be fully remyelinated. This points to the existence of remyelination mechanisms even during late stages of disease[5]. However, this and other data indicate that remyelination is incomplete in the majority of cases.

It cannot be overstated that remyelination should lead to a host of benefits to the MS patient. Remyelination can strengthen function by reestablishing electrical communication between neurons and prevent further neurodegeneration through trophic support of neurons and axons[1]. Research targeting remyelination drug therapy requires an understanding of the reasons why natural remyelination eventually fails in patients with MS.

APPROACHES IN REMYELINATION DRUG DEVELOPMENT

The timeline for taking a drug from the laboratory to the consumer market is long and costly, creating a situation of diminishing returns and leading to the focus on the repurposing of existing, proven drugs. In general, novel drugs experience 1-6 years of preclinical development, 6-11 years of clinical trials, and then 0.6-2 years for FDA approval, for a total of 7.6-19 years. Sometimes drug development occurs rapidly, as was the case of natalizumab, first identified as a potential MS treatment in 1999[6] and winning FDA approval in 2004.

Drug repurposing (also referred to as repositioning or re-profiling) utilizes existing drugs for the treatment of a disease that was not the drug's original target. These drugs have already passed through pre-clinical and clinical stages of testing examining safety profiles and tolerability, which streamlines the process to finding treatments[7]. For example, metformin, a commonly prescribed drug for diabetes, may be suggested as a chemotherapeutic agent given its association with decreased mortality in cancer patients[8]. The antimalarial drug chloroquine may have important anticancer properties[9]. First introduced in 1957 for its antiemetic and sedative properties, thalidomide has since been repurposed for multiple myeloma treatment[10]. Interestingly, dimethylfumarate was originally in use since 1959 for psoriasis[11], was repurposed for MS treatment in 2004, went straight to phase 2 trials, and recently won FDA approval for MS treatment in 2013. Thus, while there was a long delay in repurposing dimethylfumarate, FDA approval of repurposed drugs like dimethy fumarate can occur relatively rapidly after selection for MS clinical trials.

This method of borrowing from both similar and dramatically different diseases' existing drugs creates an environment where the costly overhead of research and development and clinical trials can be eliminated. Drug repurposing can be implemented in searching for a remyelination-enhancing agent in MS even many years after they are brought to market for other applications. Since many candidate drugs already exist for repurposing, high throughput screens should be particularly amenable to drug discovery. Ultimately, repurposing provides a faster and more productive way to search for and isolate a viable remyelinating agent for MS.

Though more difficult, it may also be possible to devise novel drugs that attack specific pathways involved in remyelination in MS. High throughput screens could

Table 1 Known pathways affecting remyelination in multiple sclerosis

Pathway	Potential protein targets	Drugs	Ref.
Hyaluronan	TLR2	TLR2 Ab (OPN-305)	[28]
	Hyaluronidase	Vcpal	[28,30]
Notch-1	Notch-1	(pan) BMS-906024	[14,15,17,19]
	Jagged-1	-	[14]
	Endothelin-1	PD142,893, sitaxentan, ambrisentan, atrasentan, BQ-123, zibotentan, bosentan, macitentan, tezosentan	[17]
	γ-secretase	MW167, LY450139	[93]
Retinoid X receptor	RXR-γ	Bexarotene, IRX4204	[31]
Wnt/ß-catenin	ß-catenin	PKF118-744, CGP049090, PKF118-310, XTM000990, BC21, PKF115-584, PNU-74654	[21,22]
	Tankyrases 1-2	XAV939, IWR1, JW74, JW55	[57]
	GSK3ß	SB216763	[20]
LINGO-1	LINGO-1	Anti-LINGO-1 Ab	[23-25]
CXCL12/CXCR4	CXCR7	CCX771	[37,38]
	CXCR4	AMD3100	[37,38]
GPR17	GPR17	Unnamed	Omeros

TLR2: Toll-like-receptor 2; GPR17: G protein-coupled receptor 17; CXCL4: C-X-C chemokine receptor type 4; CXCL12: Chemokine (C-X-C Motif) ligand 12.

be designed around specific components of the identified pathway as a surrogate assay for remyelination. As described below, several pathways have been identified that control remyelination in MS and relevant animal models. With characterization of the molecular components of these pathways, it may be possible to design novel drugs or repurpose old drugs targeting these pathways to enhance remyelination in MS. In a review of the literature, both novel drug discovery and existing drug repurposing are being utilized in drug development in MS.

KNOWN REMYELINATION PATHWAYS IN MS

One of the most prominent issues with inhibited remyelination is that recruited progenitors fail to mature after they are recruited to the lesion site[12]. Multiple mechanisms are proposed for the blockade of OPC maturation, including pathways involving Notch-1, Wnt, LINGO-1, hyaluronan, and Retinoid X receptor (RXR) receptor (see Table 1). One of the first pathways to be identified was involving Notch-1, which responds to a broad array of ligands including Jagged1, Delta, Contactin, and Endothelin-1[13-17]. Originally increased Jagged1 expression was noted in reactive astrocytes stimulated with TGF-ß1[14]. Borders of acute MS lesions exhibited increased expression of Jagged1, Notch-1, and inhibitory basic helix-loop-helix protein, Hes5, suggesting the presence of active Jagged1/Notch-1 signaling.

However, followup *in vivo* work was initially more contradictory, with conditional deletion of Notch-1 in PLP+ oligodendrocytes having no effect on remyelination in the cuprizone model of demyelination/remyelination[18]. Because PLP+ oligodendrocytes may be too far along in maturation to respond to Notch-1 signaling, conditional deletion of Notch-1 in CNP+ oligodendrocytes was performed, and this did in fact show precocious oligodendrocyte maturation[19]. Similarly, conditional deletion of Notch-1 in Olig2 oligodendrocytes also promoted premature oligodendrocyte maturation[19]. Remyelination was more extensive after lysolecithin demyelination in these mice as well. More recently, Endothelin-1 expression by astrocytes appears to limit oligodendrocyte through Notch-1 signaling in animal models of remyelination[17]. However, contactin is increased in MS lesions and induces myelin-associated glycoprotein (MAG) upregulation after Notch-1 stimulation[15], suggesting different opposing effects of Notch-1 on oligodendrocyte maturation. If the inhibitory effect of Notch-1 can be better characterized, there may be an opportunity to target this pathway to enhance remyelination in MS, as inhibitors to Notch, Endothelin-1 and gamma-secretase exist and have been studied in humans for other conditions (Table 1).

The canonical wnt signaling pathway has also been identified for remyelination purposes[20-22]. Although the developmental role of the wnt pathway in oligodendrocyte maturation has been known for some time, the involvement of the wnt pathway was more recently uncovered in a mouse lysolecithin injection model by in situ screens of 1040 transcription factors[21]. Three additional murine multiple sclerosis models were used to confirm the role of the wnt pathway in remyelination, including lysolecithin injection, cuprizone intoxication, and ethidium bromide injection models. TCF4 a major transcription factor involved in wnt signaling, is expressed in OPCs in demyelinated lesions. A transgenic mouse expressing dominant negative TCF4 exhibited normal OPC development but grossly impaired oligodendrocyte maturation[22]. Dominant active ß-catenin in Olg2+ cells showed decreased mature oligodendrocytes in spite of normal oligodendrocyte numbers, as well as evidence of reduced myelination in development[21]. Olig1+ specific knockout of ß-catenin also induced premature oligodendrocyte development[22]. Since Wnt pathway activation and protein expression was observed in MS lesions[21], it remains possible that the wnt pathway controls remyelination in MS.

There are multiple drugs identified that target

wnt and ß-catenin and can be assessed for effects on remyelination *in vivo*. Other targets in the wnt/ß-catenin cascade, including disheveled, axin, and porcupine, could be involved in limited OPC maturation. There are many small molecular weight drugs in clinical trials or approved for human use that modulate the functions of these proteins and wnt signaling as well, including sulindac (disheveled), bosutinib (Src kinase inhibitor) and imatinib (tyrosine kinase inhibitor) (Table 1).

Leucine rich repeat and Ig domain containing 1 (or LINGO-1) has also been shown to limit oligodendrocyte maturation and remyelination[23-25]. Upregulation and downregulation of LINGO-1 expression restricts and enhances features of oligodendrocyte maturation *in vitro*[23-25]. Functional blockade of LINGO-1 through blocking antibodies or dominant negative LINGO-1 enhances myelin sheet formation, induction of MBP expression, and myelination *in vitro*[23-25]. The repressive effect of LINGO-1 appears to work through downstream activation of a rho kinase (ROCK)[25]. *In vivo* overexpression of LINGO-1 reduces cord myelination[26]. Conversely, LINGO-1 KO mice show increased myelination in development and oligodendrocyte cultures contain an increased percentage of mature oligodendrocytes[25]. Antibody blockade of LINGO-1 enhances remyelination in EAE when given after peak disease activity, indicating an effect on remyelination processes rather than immune functions most likely[23]. Remyelination is also enhanced by anti-LINGO-1 antibody treatment in other models, including the lysophosphatidylcholine injection and cuprizone models[24]. Clearly, there is great potential for anti-LINGO-1 treatment in MS and phase 1 clinical trials are now underway (NCT01052506 and NCT01244139) (Table 1).

The glycosaminoglycan hyaluronan (HA) also inhibits OPC maturation[27,28]. Our group found that HA blocked OPC maturation in a dose-dependent manner[28]. Because specific Toll-Like-Receptor 2 (TLR2) agonists blocked OPC maturation, HA was suspected to act through TLR2 on oligodendrocytes. This suspicion was confirmed when TLR2-blocking antibodies ablated the effects of HA on OPC maturation. The downstream signaling of TLR2 was also implicated when small molecular weight inhibitors of MyD88 and IRAK1/4 also ablated the effects of HA *in vitro*. When lysolecithin and HA were injected into TLR2-null mice, enhanced remyelination occurred compared to wild-type mice. These findings indicate that HA acts on TLR2 to inhibit OPC maturation.

Our work further implicated hyaluronidase in the hyaluronan pathway. Our group suspected that high molecular weight (HMW) HA must be converted to low molecular weight (LMW) HA in order to act on TLR2, since both LMW and HMW HA block OPC maturation though only LMW is known to stimulate TLR2[28,29]. Only complete degradation of HMW HA by both hyaluronidase and ß-glucuronidase neutralized the effects of HA on OPCs by completely degrading all sources of HA. Ascorbate 6-hexadecanoate (Vcpal), a hyaluronidase inhibitor, cultured with OPCs and HMW HA allowed OPC maturation to proceed normally by limiting the conversion of HMW HA to LMW HA[28]. This effect of Vcpal was confirmed *in vivo*[30]. Based on these exciting findings, hyaluronidase inhibition should be further evaluated for remyelination effects in MS (Table 1).

Retinoid X receptor gamma (RXR-γ) has been implicated as a positive regulator in CNS remyelination[31]. RXR-γ is a nuclear receptor that dimerizes with other receptors, including retinoic acid receptors, thyroid hormone receptors, vitamin D receptors, and peroxisome proliferator activator receptors to regulate cell differentiation, proliferation, and apoptosis[32]. In MS, RXR-γ expression increases in the nuclear component of OPCs in active lesion borders but is lowered in chronic inactive lesions, suggesting RXR-γ plays a positive role in remyelination. RXR-γ is also strongly upregulated after demyelination from lysolecithin injection. Knockdown of RXR-γ expression in OPCs promotes simple undifferentiated cellular morphology compared to controls. *In vitro* testing of RXR-selective antagonists (HX531 and PA452) with OPCs reduced MBP expression. In contrast, 9-*cis*-retinoic acid, an RXR activator, increased the number of MBP+ membrane sheets in culture. *In vivo* testing showed an increased level of CC1+ differentiated oligodendrocytes and thicker myelin sheaths after 9-*cis*-retinoic acid treatment[31].

In mouse models of Alzheimer's disease, the RXR agonist bexarotene improved cognitive functioning and decreased amyloid-β plaque burden[33]. Bexarotene also has positive effects in shizophrenia[34] and cutaneous T cell lymphoma[35]. Though bexarotene nonspecifically acts on all retinoid X receptors, testing in MS patients can confirm whether bexarotene may be successful in promoting remyelination in MS. Other RXR agonists including IRX4204 may be immediately useful in clinical trials of remyelination in MS, although more preclinical work needs to be performed (Table 1)[36].

Recently, certain chemokines have been identified that modulate remyelination, although effects on inflammation are likely also involved. CXCL12 promotes remyelination by acting on the receptor CXCR4 on OPCs. The recently discovered scavenge receptor for CXCL12, CXCR7 limits the availability of CXCL12 to act on CXCR4[37]. During demyelination in cuprizone-fed animals, both receptors and CXCL12 were elevated above levels in control animals[38-41]. During remyelination phase, CXCR7 expression returns to normal while CXCR4 and CXCL12 remain elevated. CXCR7 appears to regulate the availability of extracellular CXCL12. When CCX771, a CXCR7 antagonist, was given to animals during weeks three to six (remyelination phase) of a six week cuprizone-infused diet, levels of CXCL12 and ligand activated CXCR4 were elevated in the corpus callosum. Once mice were allowed to remyelinate after cessation of the cuprizone and CCX771, mice showed significantly increased numbers of GST-pi+ (mature oligodendrocyte marker) cells and increased myelin oligodendroglial

glycoprotein (MOG) expression and myelin staining. The improvement was shown to occur through CXCR4 activation by CXCL12 when a CXCR4 antagonist blocked remyelination. Increased remyelination also correlated with CXCR4 phosphorylation[38]. In EAE, CCX771 also lead to significant decrease in peak severity of disease. The antagonist ultimately preserved axonal integrity as found through diffusion tensor imaging[37]. Antagonism of CXCR4 increased disease activity while antagonism of CXCR7 significantly decreased disease severity in the mice model of experimental autoimmune neuritis based on clinical scores[41]. Thus, treatments that either enhance CXCR4 stimulation or block CXCR7 may be useful in enhancing remyelination in MS (Table 1).

The Uracil nucleotide/cysteinyl leukotriene receptor [(also known as the G-protein coupled receptor 17 (GPR17)] is known to be involved with OPC differentiation and is activated by uracil-nucleotides (UDP-sugars) and cysteinyl-leukotrienes LTC4 and LTD4[42,43]. GPR17 mRNA levels peak in conjunction with rising MBP levels in maturing OPC cultures. In morphologically mature oligodendrocytes, GPR17 expression then decreases to very low levels. Since UDP-glucose increased the number MBP expressing cells and GPR17 expression is highest in OPCs, it was initially thought that GPR17 plays a stimulatory role in the early stages of differentiation[44]. Complicating its role in oligodendrocyte biology, GPR17 also appears to play a role in OPC migration as GPR17 antagonist prevented migration[45].

Though scientists are convinced that GPR17 activation could enhance therapy for demyelinating diseases, the role of GPR17 in remyelination is accompanied by much debate because GPR17 expression and activation is also thought to arrest progenitors in a premature state. GPR17 overexpressing transgenic mice showed significant reduction in MBP and PLP1 expression. Prolonged overexpression in the transgenic mice resulted in differentiation arrest or apoptotic cell death compared to WT animals[46]. These *in vivo* data countered understanding that GPR17 plays a positive role in OPC maturation and instead supported the idea that GPR17 arrests CNS cells at a pre-myelination stage. However, efforts towards creating a safe and effective GPR17 antagonist were put into motion once the GPR17 activator MDL29951 inhibited oligodendrocyte maturation in culture[47]. As part of its G-protein coupled receptors (GPCR) program, the biopharmaceutical company Omeros significantly increased mean clinical scores using GPR17 antagonists in EAE animals. Identification and evaluation of GPR17 modulators in multiple remyelination models is needed to fully evaluate for remyelinating effects (Table 1).

Other factors also play a role in remyelination and may inhibit oligodendrocyte maturation. Chondroitin sulphate proteoglycans (CSPGs) that exist on the surface of terminally differentiated oligodendrocytes inhibit growth responses using the Rho/ROCK/LIMK cascade[48]. Klotho expression in brain decreases with age and enhances oligodendrocyte maturation as well as cognitive benefits[49]. Axonal damage can also result in the accumulation of myelin-associated inhibitors or myelin debris that inhibits OPC differentiation[50,51]. Efficient phagocytic removal of myelin debris is required for remyelination to occur[50,52]. The extent of overlap among these mechanisms is still unclear. Furthermore, drugs that modulate these additional pathways could be discovered through high throughput screens, such as with Klotho[53].

HIGH THROUGHPUT SCREENS FOR REMYELINATION INDUCING DRUGS

High throughput screening is a rapid method for identifying drugs that may be useful in treating disease. One of the difficulties in remyelination research is what screens would be useful for screening and modeling aspects of remyelination. Initial screens using zebrafish were complicated by multiple effects of drugs on OPC proliferation (olig2 counts) as well as maturation (MBP expression)[54]. With this screen several drugs were identified that increase OPC proliferation but none that enhanced maturation. Since OPC maturation is essential for remyelination and may be blocked in MS, high throughput screens utilizing OPC cultures examining markers of maturation has been identified as a more streamlined method of identifying drugs. Several drugs have been discovered using this approach recently, including benztropine[55] (Table 2).

Through use of an OPC maturation screen, benztropine was found to be a potent inducer of differentiation based on the expression of MBP and MOG *in vitro*[55]. Benztropine is a muscarinic receptor antagonist used for the treatment of Parkinson's disease and readily crosses the blood brain barrier (BBB). Cells expressed the highest level of MBP after treatment given at an immature state suggesting that the compound acts most effectively on the cells at an early stage of differentiation. In cuprizone induced demyelination and in EAE experiments, benztropine improved clinical outcomes and myelin content indicating enhancement of remyelination rather than immune effect[55].

However, benztropine is associated with dose-dependent side effects including tachycardia, paralytic ileus and urinary retention[55,56]. Although benztropine may need to be dosed at unsafe levels to be effective in MS remyelination, clinical trials in MS patients could help determine whether a safe dosage effective in promoting remyelination does indeed exist.

Based on the findings that oligodendrocytes can myelinate paraformaldehyde fixed axons as well as electron-spun nanofibers, micropillar arrays were fabricated to assess myelination drugs in a high throughput format[57] (Table 2). OPCs bound to micropillars and matured into oligodendrocytes that wrapped the micropillars. Similar to the work of Deshmukh *et al*[55], this group found a cluster of antimuscarinic compounds including benztropine that enhanced oligodendrocyte

Table 2 Drug classes identified in remyelination screens

Drug class	Compounds	Ref.
Adrenergic agonist	Methoxamine, nerepinephrine, tolaxoline, salmeterol	[55]
Dopamine antagonists	Opipramol, flupentixol, fluphenazine, trifluoperazine, perphenazine, quetiapine	[55,57]
Dopamine uptake inhibitor/dopamine agents	Vanoxerine, GBR12935, methyldopa	[55]
Histamine antagonists	Clemastine, doxylamine, clemizole	[55,57]
Adrenergic antagonist	Opipramol, trifluoperazine, Cgp-26505, tolazoline, quetiapine	[55,57]
Anticholinergics/cholinesterase reactivators	Homatropine, clemastine, benztropine, disopyramide, trospium, diacetyl-monoxime, tiotropium, oxybutynin, atropine, ipratropium, hyosamine, atropine, methy atropine, octatropine, glycpyrrolate, carbetapentane, piperildolate, bevonium, propiverine, dicyclomine, mepenzolate, trihexylphenidyl	[55,57]
Phosphodiesterase inhibitor	8-bromo cyclic GMP, IBMX, enprofylline, enoximone, rolipram	[55]
PPAR agonist	GW-1929	[55]
Ion channel blockers	Gabapentin, 8-aminobenzoic acid, disopyramide, oubain	[55]
Serotonin modulators	Brl-15572, paroxetine, clemizole, quetiapine	[55,57]
Glutamate receptor antagonist	UPF-523, l-aminodan-1,5-dicarboxylic acid	[55]
Beta-catenin inhibitor	XAV939	[55]
Retinoic acid receptor agonist	Retinoids, AM580	[55,94]
Thyroid hormone receptor agonist	T3	[55,57]
COX-2 inhibitors	Niflumic acid, flunixin	[55]
HMG-CoA reductase Inhibitor	Mevastatin, simvastatin	[55]
Rho kinase inhibitor	Ml-7, Ml-9, fasudil	[55,63,94]
Antifungal/antibacterial	Ketoconazole	[55]
Cathartic/emetic	Emetine	[55]
Opioid antagonists	Levallorphan	[55]
Glucocorticoids	Dexamethasone, hydrocortisone, budesonide, flunisolide	[94]
EGF/ErbB2 inhibitor	PD174265	[94]

PPAR: Peroxisome proliferator activator receptor. COX-2: Cyclooxygenase-2; HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A; EGF: Epidermal growth factor.

maturation and wrapping. Clemastine, an antihistamine compound with antimuscarinic properties, and quetiapine, an atypical antipsychotic, were also identified.

Through use of a DRG/oligodendrocyte coculture system, clemastine and benztropine were both shown to enhance myelin formation. After lysolecithin-induced demyelination, clemastine enhanced remyelination of cord lesions. Since clemastine is a safe over the counter drug (Tavist), clemastine is an exciting prospect for remyelination in MS. A phase 2 clinical trial has just begun (NCT02040298) and aims to study improvement in visual evoked potentials as well as myelin water volume and magnetization transfer ratios by MRI. Interestingly, GlaxoSmithKline is also conducting a phase 2 clinical trial (NCT01772199) largely in Europe that examines the effect of a histamine 3 receptor antagonist GSK239512 on remyelination in MS also through changes in magnetization transfer ratios. Further use of these high throughput screens may continue to identify additional targets beyond antimuscarinic (benztropine), antihistamine (clemastine), and antidopamine (quetiapine) pathways.

DRUGS IN SEARCH OF A MECHANISM

Another approach to generating drugs to help remyelinate MS brains has been a shotgun approach of generating, isolating, and identifying monoclonal antibodies with remyelinating effects. Beginning in 1987, the possibility was explored that monoclonal antibodies generated against myelin antigens might have a reparative and remyelinating effect. This work has continued to the present and has generated at least one viable antibody rHIgM22. However, a flaw in this approach is that mechanism of action is often hard to identify, as has been the case with rHIgM22.

In 2000, this line of work identified several human IgM antibodies, including rHIgM22, that enhance myelin formation from patients with Waldenstrom's macroglobinemia[58]. Once *in vivo* data confirmed its reparative properties, rHIgm22 became a very promising solution for impaired remyelination in MS because of its potency and safety. Virus-induced Theiler's murine encephalitis animals significantly reduced in clinical severity with rHIgM22 treatment at the smallest effective dose of 500 ng[59]. Volumetric measurements of spinal cord lesions revealed reduction in lesion size by 83% of all lesions. Biotinylated rHIgM22 was also able to pass the blood-brain-barrier in animals[60]. The half-life of rHIgM22 was determined to be 15.4 h and the antibody was cleared form the systems of animals within a short 48 h[61]. The short half-life and small effective dose of rHIgM22 amplified excitement for a drug with a low probability of causing adverse effects.

The mechanism through which rHIgM22 works is still unknown though *in vitro* data has identified key players working in conjunction with rHIgM22. rHIgM22 binds to the surface of oligodendrocytes though exactly what the antibody binds to is unknown[61]. In mature oligodendrocyte cultures, rHIgM22 highly co-localized with the αv integrin β3 and partially co-localized with integrin β1. Integrins are cell surface

Table 3 Repurposed drug potential for remyelination

Drug	Safety	BBB	*In vitro* effects	*In vivo* effects	MS trials	Mechanism
Quetiapine	+	+	+	+	NCT02087631	Atypical antipsychotic
Fasudil	+	NA	+	+		ROCK Inhibitor
Olesoxime	+	+	+	+	NCT01808885	Mitochondrial permeability transition pore modulator
Ibudilast	+	+	+	+	NCT01982942 NCT01910259	Phosphodiesterase PDE4 inhibitor
Simvastatin	+	+	+/-	+/-	NCT00647348	HMG-CoA reductase inhibitor
Lovastatin	+	+	+	+		HMG-CoA reductase inhibitor
Clemastine	+	+	+	+	NCT02040298	Antihistamine
IRX4204	NA	+	+	NA		Retinoid X receptor agonist
Bexarotene	+/-	+	-	-		Retinoid X receptor agonist
Benztropine	+/-	+	+	+		M1/M3 muscarinic receptor antagonist

BBB: Blood brain barrier; ROCK: Rho kinase; NA: Not available; MS: Multiple sclerosis; HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A.

proteins that are involved with signaling for cell migration and differentiation. rHIgM22 also blocked oligodendrocyte apoptosis in culture based on reduction of caspase 3 and caspase 9 cleavage. Decreased MBP and MOG expression *in vitro* clarified rHIgM22's primary mechanism, which is counterintuitive. rHIgM22 primarily provides protection from cell death while also delaying differentiation. The delayed differentiation is thought to occur through growth factor mediated inhibition that keeps cells in a proliferative state[61]. Finally, it was recently found that rIgM22 effects require PDGF to increase OPC proliferation[62]. While its *in vivo* data seem very promising, more work is required to establish mechanism of action for rIgM22. Results from the ongoing rHIgM22 phase 1 clinical trial will also begin in early 2015 (Acorda Therapeutics).

REPURPOSING DRUGS FOR REMYELINATION

Discovery of remyelination drugs may be rational by dissection of known pathways, may be through drug screens, but may also be through a more serendipitous pathway. We discuss here known drugs studied for other diseases or conditions that were also assessed for effects in remyelination or MS models. While there may be others, we will discuss fasudil, quetiapine, ibudilast, and olesoxime. Simvastatin and lovastatin are possibly also useful in remyelination although they may benefit MS outcomes through other mechanisms (Table 3).

Fasudil is ROCK inhibitor used for the treatment of vascular disease[63]. ROCK-II, the downstream effector of RhoA, phosphorylates molecules responsible for actin filament regulation[64]. Fyn-1 kinase acts on the GTPase RhoA which plays a role in oligodendrocyte morphology[65]. When OPCs were cultured with fasudil and myelin protein extracts inhibitory to oligodendrocyte differentiation, oligodendrocyte differentiation proceeded more rapidly[63]. In the presence of myelin protein extracts, neonatal rat OPCs showed decreased activation of Fyn-1 and increased levels of both GTP bound RhoA and activated ROCK-II[48]. siRNA mediated gene silencing or inhibitors of the RhoA- ROCK-II pathway induced OPC differentiation in the presence of inhibitory myelin debris[63]. It is also important to note that the ROCK inhibitors Y-27632, fasudil and dimethylfasudil increased neurite outgrowth dose-dependently in neurons cultured with CSPGs[48,66]. Thus, ROCK inhibitors including fasudil may benefit MS in that these treatments may induce more remyelination as well as axonal regrowth and neuroregeneration.

Another medication that should be evaluated for repurposing for MS remyelination is quetiapine, an atypical antipsychotic approved for the treatment of schizophrenia and acute bipolar disorder[67,68]. *In vitro* data shows that quetiapine facilitates oligodendrocyte lineage development[69]. The cuprizone animal model treated with quetiapine, though analyzed for locomotive and hyperactive indicators of schizophrenia, showed increased MBP expression positively correlated with length of time the drug was administered post-cuprizone recovery. Quetiapine increased the number of mature oligodendrocytes in treated animals compared to vehicle-treated controls suggesting quetiapine enhances both oligodendrocyte maturation and survival[70]. Apart from sedative effects, this drug has been generally well tolerated in patients with Parkinson's and Alzheimer's disease but clinical trials are needed to test safety, tolerability and efficacy in patients with MS[71,72]. A phase I/II trial is underway in Canada for this purpose (NCT02087631).

Ibudilast is an anti-inflammatory drug used in the treatment of asthma and stroke in Japan. Because of its anti-inflammatory effects, Ibudilast was first investigated for effects in EAE[73]. Prophylactic ibudilast treatment ameliorated severity of EAE but did not modify disease if given after disease onset. Mechanism of action appeared to be through limiting inflammatory infiltrate with mild suppression of T cell proliferation in regional lymph nodes. However, a phase 2 trial in relapsing remitting MS showed no beneficial effect of ibudilast both in terms of relapse rate and formation of new MRI lesions in a 12 mo interval[74]. A neuroprotective effect was postulated since a significant reduction in brain atrophy and in number of persistent T1 "black holes" was observed. No analysis was possible

to differentiate neuroprotective, neuroregenerative, or remyelinative effects.

More recent data also indicate rolipram, a PDE4 phosphodiesterase inhibitor like ibudilast, promotes OPC differentiation and remyelination *in vivo*[51]. Rolipram is postulated to negate effects of myelin protein extracts on inhibiting remyelination. Unfortunately, rolipram was ineffective and poorly tolerated in phase 2 relapsing remitting MS trials[75]. Since ibudilast is more potent than rolipram in PDE4 inhibition, there is hope that ibudilast will be more effective at more tolerable dosing. Several phase 2 trials are underway in MS patients, including a NeuroNext trial (NCT01982942) and the MS-SMART trial in England (NCT01910259) that will examine amiloride, rilozole, and ibudilast. Both trials will examine magnetization transfer ratio by MRI and this may detect effects on remyelination.

A HMG-CoA reductase inhibitor used for hypercholesterolemia, simvastatin has had mixed results in remyelination studies. Simvastatin promoted elaboration and extension of OPC and oligodendrocyte processes followed by process retraction days after. Longterm simvastatin treatment of OPCs worsened cell process elaboration. Cell process retraction could however be rescued by the addition of cholesterol[76]. Simvastatin use in cuprizone models of remyelination also raised concerns about deleterious effects of simvastatin on remyelination. After demyelination by cuprizone, animals treated with simvastatin exhibited significantly less remyelination compared to controls. Simvastatin treatment appeared to maintain OPCs in an immature state with no apparent effects on overall OPC numbers. Overall, simvastatin did not promote maturation and may have even been deleterious in the cuprizone model.

Cholesterol depletion as a consequence of using simvastatin could disrupt the functioning of lipid rafts that play a role in the remyelination process[77]. Simvastatin belongs to the group of lipophilic statins that are known to reduce levels of the lipid raft marker flotillin; decreased flotillin suggests that raft-associated proteins cannot access the membranes after statin treatment[78]. Because of promising anti-inflammatory response in EAE animals[79], simvastatin did however move towards clinical trials mainly in secondary progressive MS[80]. In a 2004 study, RRMS patients were given a daily 80 mg dose of simvastatin over the course of six months. MRI analysis concluded that Gadolinium-enhancing lesion volume shrank by an average of 41% after treatment[81]. A more recent secondary progressive study on simvastatin (MS-STAT; NCT00647348) showed for the first time a dramatic benefit in clinical and MRI outcomes[82]. The average brain atrophy rate significantly decreased in the simvastatin-treated group. Expanded Disability Severity Scale scores at 24 mo were also lower than placebo (average 5.93 *vs* 6.35)[82]. At present, it is not entirely clear the mechanism through which patients on simvastatin benefited, although anti-inflammatory effects are most likely.

Another blood-brain barrier permeable lipophilic statin, lovastatin may also be considered for MS clinical trial testing. In EAE animals treated with lovastatin, MBP, proteolipid protein (PLP), MOG, and MAG expression increased compared to EAE controls though expression did not match the healthy controls[83]. Lovastatin also reduced gadolinium-enhancing lesion load in MS patients, suggesting an anti-inflammatory effect[84]. Although there is some controversy about effects on myelin formation, the statin class of drugs may provide neurologists with orally deliverable agents for secondary progressive MS and possibly for remyelination.

Olesoxime has just recently been shown to prevent progressive loss of motor function in individuals with spinal muscular atrophy and could also be considered for the treatment of MS. Olesoxime has shown promise by increasing the area of myelinated axons in mouse forebrain slice cultures by approximately 40%[85]. In the same study, healthy new born and adult mice fed olesoxime had increased numbers of oligodendrocytes in the corpus callosum along with increased numbers of myelinated axons in the region. Myelin sheath thickness increased during neonatal development when animals were treated with olesoxime. Mice were also put on a cuprizone diet to test the effects of olesoxime on the demyelination and remyelination phases in animals. At peak demyelination, treated groups had higher MBP and NF (neurofilament) content. When animals were evaluated two weeks after peak demyelination, analysis showed that proliferation was promoted during remyelination based on increased Olig2+ cell counts; a two-fold increase in myelinated axons compared to vehicle-treated animals was found as well. Animals pretreated with olesoxime food pellets were injected with lysolecithin to induce focal demyelination; in this model, expression of differentiation protein markers did not differ between the treated and untreated group though CC1+ mature oligodendrocytes were higher in number in the pretreated group. MRI analysis of the lysolecithin/olesoxime pretreated animals showed attenuation, though insignificant, of lesions in the treated group. Olesoxime is currently undergoing a phase 1b trial in MS patients as add-on therapy to the immunosuppressant interferon-beta (MSREPAIR; NCT01808885) and diffusion tensor imaging and magnetization transfer ratio will be performed to assess remyelination.

Two other drugs, minocycline and rolipram were to date unsuccessful in MS trials. Minocycline is a tetracycline antibiotic that easily crosses the blood–brain barrier and has been used to treat a variety of infections for years[86]. In a cuprizone-fed animal model, minocycline inhibited microglial activation that suppressed expression of ciliary neurotrophic transcription factor. However, the drug also decreased the number of CC1+ mature oligodendrocytes in animals[87]. In clinical trials with Rebif, minocycline use was associated with increased brain atrophy and progression and the trial was halted. Minocycline use with glatiramer showed no benefit over glatiramer alone

in MS patients as well[88].

Rolipram was originally tested for treatment of depression though not tolerated well in clinical trials. Nausea was reported as the main side effect which limited its clinical application[89,90]. When considered as a treatment option for MS, rolipram showed a dose dependent increase in MBP+ oligodendrocytes *in vitro*. After rolipram treatment, lysolecithin injected animals showed increased MAG, CNPase, and MBP expression[91]. Myelin sheaths in ethidium bromide injected animals were found to be thicker when analyzed fourteen days after lesion[51]. Positive *in vitro* and *in vivo* results propelled rolipram to a phase I/II clinical trial in a small group of MS patients. The measurement of contrast-enhancing lesions (CELs) from MRI results indicated that brain inflammatory activity actually increased with rolipram treatment. The total number of CELs per patient per month significantly increased in the treatment group. Adverse events significantly increased and exacerbations generally increased with treatment[75]. Though originally promising, rolipram dosing may not be high enough for remyelination to occur in humans. Nevertheless, rolipram may be worthy of further research efforts since both rolipram and ibudilast are phosphodiesterase inhibitors and appear to enhance remyelination in animal models.

Clearly several extant drugs could potentially be repurposed for remyelination in MS. One issue cogently raised by others in relation to the MS-STAT trial[92], is the difficulty that exists in performing required phase 3 clinical trials to validate these drugs for remyelination in MS. The patents of many of the drugs named here have expired so pharmaceutical companies are expected to have limited interest in financing expensive trials. Since no trial has actually produced positive data showing evidence of remyelination in MS, the risk is also especially high to any trial studying remyelination. Alternative funding mechanisms do not exist since NIH and other governmental agencies have no established mechanism through which a large-scale expensive trial can be performed. Furthermore, medical insurance companies may not pay for treatments only characterized by limited phase 2 trials utilizing approximately 100 patients. It remains to be seen how best to fund phase 3 trials of this sort but it is likely trials of newer and more patentable drugs will lead the way first.

CONCLUSION

Remyelination therapeutics is an emerging and exciting field in MS drug development. While it is important to remember sustained neurological deficits in MS are clearly related to both neurodegeneration as well as impaired myelination, remyelination-enhancing treatments may improve patients' function and quality of life in spite of their restricted effects. Several remyelination pathways important to MS have been identified, including those of LINGO-1, hyaluronan, Notch-1, RXR receptor, and wnt/ß-catenin. Other newer discoveries include the pathways involving CXCL12/CXCR4 and GPR17, and the involvement of Endothelin-1 in the Notch pathway. A number of known drugs with effects on these pathways can be evaluated for remyelination enhancing effects in MS, although this has yet to occur. High-throughput screens have identified multiple antimuscarinic drugs with good remyelination potential but use of at least benztropine may be problematic due to dose limiting side effects. Also identified by screens, clemastine, with similar antimuscarinic but also antihistamine effects, may be useful in remyelination in MS. Other drugs identified through other means such as rHIgM22 are in the beginning stages of clinical trials. Drug repurposing, through screens or more serendipitously, has found that many drugs could enhance remyelination, including benztropine, clemastine, quetiapine, fusadil, olesoxime, and ibudilast, among others. Many other these identified drugs are undergoing clinical trials, some with endpoints that examine remyelination. Difficulties exist in design of clinical trials to identify remyelination in a cost-efficient, sensitive, and reproducible manner. In addition, funding for clinical trials of repurposed drugs may be difficult to acquire, which may lead clinicians and insurers to an uncertain position of whether to use certain treatments. However, with recent dramatic advances in remyelination research, we are optimistic that many new remyelination treatments for MS will arise and be in use in the next decade or so.

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REVIEW

Prevalence, clinical features and treatment of depression in Parkinson's disease: An update

Omar ME Abdel-Salam

Omar ME Abdel-Salam, Department of Toxicology and Narcotics, Medical Division, National Research Centre, Cairo 12311, Egypt
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Correspondence to: Omar ME Abdel-Salam, MD, PhD, Department of Toxicology and Narcotics, Medical Division, National Research Centre, Tahrir St., Dokki, Cairo 12311, Egypt. omasalam@hotmail.com
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Abstract

Parkinson's disease (PD) is one of the most prevalent neurodegenerative diseases which typically affects individuals over 65 years. Although the symptomatology is predominantly motor, neuropsychiatric manifestations, *e.g.*, depression, apathy, anxiety, and cognitive impairment occur in the course of the illness and can have a great impact on the quality of life in these patients. Parkinson's disease is commonly comorbid with depression with prevalence rates of depression, generally higher than those reported in general population. Depression in PD is frequently underestimated and consequently undertreated, which have significant effects on the quality of life in these patients. The neurobiology of depression in PD is complex and involves alterations in dopaminergic, serotonergic, noradrenergic and possibly other neurotransmitter systems which are affected in the course of the disease. The tricyclic antidepressants and the selective serotonin reuptake inhibitors are the two classes of antidepressant drugs used for depressive symptoms in PD. Several published studies suggested that both classes are of comparable efficacy. Other serotonergic antidepressants, *e.g.*, nefazodone and trazodone have also been of benefit. Meanwhile, there are limited data available on other drugs but these suggest a benefit from the serotonin and noradrenaline reuptake inhibitors such as mirtazapine, venlafaxine, atomoxetine and duloxetine. Some of the drugs used in symptomatic treatment of PD, *e.g.*, the irreversible selective inhibitors of the enzyme monoamine oxidase-B, rasagiline and selegiline as well as the dopamine receptor agonist pramipexole are likely to have direct antidepressant activity independent of their motor improving action. This would make these drugs an attractive option in depressed subjects with PD. The aim of this review is to provide an updated data on the prevalence, clinical features of depression in subjects with PD. The effects of antiparkinsonian and antidepressant drugs on depressive symptoms in these patients are also discussed.

Key words: Antidepressant drugs; Depression; Serotonin reuptake inhibitors; Parkinson's disease; Tricyclic antidepressants

Core tip: The development of depressive symptoms in Parkinson's disease (PD) has important implications on the daily functioning and quality of life. It is thus important to diagnose and treat depression effectively in these patients. This review aims to discuss the

prevalence, associated factors and drugs used to treat depressive symptoms in PD.

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INTRODUCTION

Idiopathic Parkinson's disease (PD), also known as primary PD or paralysis agitans is the second most common neurodegenerative disease[1]. The disease affects about 1% of the population over the age of 65 years[1,2]. Estimates of the prevalence of PD in Europe vary from 65.6-12500 per 100000, while annual incidence estimates vary from 5-346 per 100000[3,4]. The disease is characterized by a triad of motor symptoms, bradykinesia, rigidity and resting tremors[5]. These symptoms result from the loss of dopaminergic neurons in the substantia nigra pars compacta, with consequent depletion of dopamine in the striatum[6,7]. Other neuronal populations are also affected including serotonergic, noradrenergic and cholinergic systems, which contributes to the development of non-motor symptoms during the course of the disease[8]. Neuropsychiatric symptoms such as depression, apathy, anxiety, sleep disturbances, cognitive impairment occur in the premotor or presymptomatic phase of the disease, as well as in the advanced disease, and can substantially affect the quality of life and activities of daily living[9-11]. The pathophysiology of these symptoms is complex, and reflects the widespread cortical and brainstem pathology and affection of several neurotransmitter pathways[12]. Depression is particularly common in PD patients, is frequently overlooked, and is known to cause significant morbidity[13]. In this paper it is aimed to provide a comprehensive and an updated account on the prevalence and clinical features of depression in subjects with PD. The effect of antiparkinsonian drugs on the course of depression in these patients as well as the tolerability and efficacy of antidepressant medications are presented. Non-pharmacological approaches to treat depression in patients with PD are also discussed.

DEPRESSION

Mood disorders can be subdivided into: (1) unipolar (depressive) disorders; (2) bipolar disorders (formerly manic-depressive illness); and (3) other mood disorders, *e.g.*, psychotic mood disorders, postpartum mood episodes with psychotic features, mood disorders due to a general medical condition, and substance/medication-induced mood disorder[14-16]. The term depression describes a range of mood disturbance in the form of an unhappy or sad mood to markedly decreased mood[16]. There are two diagnostic classifications for depressive disorders. One is the "International Classification of Disorders", 10th edition (ICD-10) system of the World Health Organization[14]. The other is the "Diagnostic and Statistical Manual of Mental Disorder", 5th edition (DSM-V) from the American psychiatric association[15]. The diagnosis of major depression illness depends on the presence of a number of symptoms that include depressed mood, loss of interest or pleasure, significant weight loss, or weight gain, sleep disturbances, psychomotor agitation or retardation, fatigue, loss of energy, feelings of worthlessness or excessive or inappropriate guilt, diminished ability to think or concentrate, or indecisiveness, recurrent thoughts of death or suicidal ideation. One of the symptoms should be either depressed mood or loss of interest or pleasure in usual activities (DSM-V)[15] (Table 1). Symptoms must have been present almost every day for a minimum of 2 wk, represent a change from previous functioning, result in clinically significant distress or impairment in social, occupational, or other important areas of functioning, and are not due to a medication, substance abuse or a general medical condition.

Other types of depressive disorders include disruptive mood dysregulation disorder, persistent depressive disorder (dysthymia), premenstrual dysphoric disorder, substance/medication-induced depressive disorder, depressive disorder due to another medical condition, other specified depressive disorder, and unspecified depressive disorder[15]. Depression might occur in the sitting of medical diseases, especially chronic illnesses such as diabetes mellitus, congestive heart failure, myocardial infarction, rheumatologic disorders. Depressive symptoms can be associated with other psychological diseases, including psychotic disorders[17] or caused by a number of medications including β-adrenoceptor blockers, α-adrenoceptor blockers, digoxin, calcium channel blockers, methyldopa, corticosteroids, psychostimulants , isotretinoin, and interferon-α[18-21]. Depressive symptoms are also a common and often characteristic feature in a number of neurological disorders such as stroke, PD, multiple sclerosis, or epilepsy, in which depression has a strong impact on both quality of life and outcome of the primary neurological disorder[22].

PREVALENCE AND FEATURES OF DEPRESSION IN PD

The estimated lifetime prevalence of depression in the general population is approximately 17%-20%[23,24]. It is estimated that up to 85% of patients will have more than one episode in their lifetimes[23,25]. Moreover, up to 20% of patients with depression will have symptoms lasting for 2 years or more, *i.e.*, chronic depression[23,25]. Prevalence estimates for depression

Table 1 Diagnostic criteria for major depressive episode

Depressed mood most of the day, nearly every day
Markedly diminished interest or pleasure in all or almost all activities
Significant weight loss when not dieting or weight gain
Insomnia or hypersomnia nearly every day
Psychomotor agitation or retardation
Fatigue or loss of energy
Feelings of worthlessness or excessive or inappropriate guilt
Diminished ability to think or concentrate, or indecisiveness
Recurrent thoughts of death or suicidal ideation

Five or more of the symptoms must have been present during the same 2-wk period, represent a change from previous functioning and cause significant distress or impairment in social, occupational or other functioning. One of the symptoms should be either depressed mood or loss of interest or pleasure, significant weight loss or weight gain. Symptoms should be present nearly every day, most of the day are not due to drug or a general medical condition. Criteria adapted from Diagnostic Statistical Manual of Mental Disorders[15].

in PD vary in different studies, but are clearly higher than those reported in general population. Studies in general report a prevalence rate between 21% and 40%[26-32]. Prevalence rates as low as 2.5% and as high as 66% have also been reported[33-52] (Table 2). It has been found that persons treated with antiparkinson drugs had significantly increased rate of subsequent antidepressant drug treatment compared with controls, indicating the high frequency of depression in PD[53]. Moreover, initiation of any antidepressant drug therapy was associated with a higher risk of PD in the 2 years from the beginning of treatment. This suggested that depressive symptoms were an early manifestation of PD, before the appearance of motor symptoms[54]. Jasinska-Myga *et al*[36] found that 72% of patients developed depression within ten years of symptomatic PD onset (mean time to depression: 7.9 years). Becker *et al*[55] reported approximately twofold increased risk of developing depression in PD patients when compared to PD-free population.

Depression itself might be an independent risk factor for developing PD. This is because in patients with depression followed up for 10 years, 1.42% developed PD compared with 0.52% in the control group. In this study, patients with depression were 3.24 times more likely to develop PD compared with the those of the control group[56]. Moreover, patients with psychiatric illnesses exhibited 2.38-fold increased risk for developing PD compared with nonpsychiatric individuals. The highest risk for developing PD was observed in patients with schizophrenia[57]. The risk of the onset of major depression is influenced by genetic factors. This is due at least in part to the effect of genetic factors in modulating the individual's response to the depression-inducing effect of stressful life events[58]. Vanderheyden *et al*[37] found that 30% of PD patients had a history of mood disorder and 46% were prescribed an anxiolytic, an antidepressant, or an atypical neuroleptic, or a combination of these drugs. A study on first-degree relatives of patients with PD showed increased risk of depressive and anxiety disorders compared with first-degree relatives in the control group[59]. Puschmann *et al*[60] described a family with mild and slowly progressive L-dopa responsive autosomal dominant PD whose members also had depression. This suggests a common genetic vulnerability for mood disorders and PD.

The clinical manifestations of PD depression include apathy, psychomotor retardation, memory impairment, pessimism, irrationality, and suicidal ideation without suicidal behavior[61]. Depressed PD patients share many features that are present in depressed subjects without PD such as apathy, loss of initiative and decisiveness, insomnia, lack of energy and fatigue. The clinical spectrum of depression in PD patients differs in that features such as anhedonia, sadness, feelings of self-blame, feelings of guilt, sense of failure, self-destructive thoughts, suicide or suicidal ideation are much less common compared to patients with major depression not having PD. Concentration problems, however, are more common compared with depressed control subjects[62,63]. Suicidal ideation is not only a feature of major depression illness but also occurs in other neurological diseases, *e.g.*, multiple sclerosis, epilepsy, Huntington's disease, and PD. The most common risk factors being hopelessness, depression, and social isolation[64-66]. Major depression is a major risk factor for suicide and suicidal acts, which usually occur during major depressive episodes or mixed episodes[67]. Sokero *et al*[68] found that during the current major depressive episode, 58% of all patients had experienced suicidal ideation and 15% had attempted suicide most of whom (95%) had also had suicidal ideation. The severity of depression and current alcohol dependence or abuse was among factors that predicted suicide attempts. In their study, Subramaniam *et al*[69], reported that the prevalence of suicidal ideation, plan, and attempt to commit suicide among patients with lifetime major depressive illness was 43.6%, 13.7% and 12.3%, respectively. Inagaki *et al*[70] reported current suicidal ideation in 71.4% of patients with major depressive illness.

Suicide or death ideation are also common in depressed patients with PD. Kostić *et al*[71] found that in PD patients, followed for 8 years, the suicide-specific mortality was 5.3 times higher than expected. Current death and/or suicidal ideation were present in 22.7% of the patients. Major depression, psychosis, and hopelessness were associated with such ideation. Nazem *et al*[72] found that death ideation or suicide ideation were present in one-third of the sample, and 4% had a lifetime suicide attempt; increasing severity of depression, impulse control disorder, and psychosis were associated with either ideation. A lifetime prevalence of suicidal ideation in 11.6% of PD patients was also reported; the presence of depression and history of impulse-control disorder behaviors were important risk factors[73]. Other workers reported a prevalence of suicidal ideation in 14.4% of their sample but no attempted suicide; major depression being the

Table 2 Classification, mechanism of action and dosage range of antidepressants

Class	Mechanism of action	Generic name (trade name)	Dose range (mg/d)
Older antidepressants			
Mixed serotonin and norepinephrine reuptake inhibitors			
First-generation tricyclic antidepressants	Inhibit neuronal reuptake of norepinephrine and serotonin	Amitriptyline (elavil)	100-300
		Clomipramine (anafranil)	100-250
		Doxepin (adapin)	100-300
		Imipramine (tofranil)	50-300
		Trimipramine (surmontil)	100-300
		Protriptyline (vivactil)	75-200
		Lofepramine	15-60
Second-generation tricyclic antidepressants	Inhibit neuronal reuptake of norepinephrine and serotonin	Desipramine (norpramin)	100-300
		Nortriptyline (pamelor)	50-150
Tetracyclic antidepressants	Inhibit neuronal reuptake of norepinephrine and serotonin	Maprotiline (ludiomil)	100-200
		Amoxapine (asendin)	50-300
Heterocyclic agents Triazolopyridines	Mixed serotonin effects: Serotonin (5-HT2A) receptor blockade with serotonin reuptake inhibition	Trazodone (desyrel)	150-400
Monoamine oxidase inhibitors	Nonselective inhibitor of monoamine oxidase A and B	Phenelzine (nardil)	60-90
		Tranylcypromine (parnate)	20-60
		Selegiline (eldepryl)	5-10
Newer antidepressants			
Selective serotonin reuptake inhibitors	Selectively inhibit the reuptake of 5HT at the presynaptic neuronal membrane. Sertraline also markedly inhibits dopamine reuptake	Fluoxetine (prozac)	20-60
		Fluvoxamine (luvox)	100-300
		Paroxetine (paxil)	20-50
		Sertraline (zoloft)	50-200
		Citalopram (celexa)	20-40
		Escitalopram (lexapro)	5-20
Serotonin and noradrenaline reuptake Inhibitors	Potent inhibitors of 5HT and norepinephrine uptake; weak inhibitors of dopamine reuptake	Venlafaxine (effexor)	75-350
		Milnacipran (savella)	12.5-100
		Duloxetine (cymbalta)	60
Norepinephrine reuptake inhibitors	Noradrenaline reuptake inhibitor. Inhibits norepinephrine reuptake without inhibiting serotonin reuptake	Viloxazine	150-300
		Reboxetine (edronax)	4-8
		Atomoxetine (strattera)	40-80
Reversible inhibitors of monoamine oxidase A	Selective, reversible inhibitors of monoamine oxidase A: resulting in increased concentrations of NE, 5-HT, and dopamine in synapse	Moclobemide	300-600
		Brofaromine	75-150
5HT2 receptor antagonists/reuptake inhibitor serotonin modulators	Mixed serotonin effects. Inhibition of the reuptake of serotonin and selective postsynaptic 5-HT2A blockade	Nefazodone (serzone)	300-600
		Desvenlafaxine (pristiq)	50 mg once daily
		Ritanserin	5-10
5HT1a receptor agonists	Partial agonist of serotonin 5-HT1a	Gepirone, ipsapirone, tandospirone, felsinoxan	
α2-noradrenergic antagonists	Complex action on serotonin and noradrenaline *via* Serotonin (5-HT2A and 2C) receptor blockade and presynaptic α2-receptor blockade	Mirtazapine (remeron)	15-45
GABA-mimetics	GABAA and GABAB receptor agonists	Fengabine	900-1800
Dopamine reuptake inhibitors	Increases activity of norepinephrine and dopamine only; no significant effect on serotonin	Buproprion (wellbutrin)	200-450
Melatonin receptor agonists	Melatonin MT1 and MT2 receptor agonist and serotonin 5HT2C receptor antagonist	Agomelatine (valdoxan)	25-50
Herbal remedy: *Hypericum perforatum*/ St. John's wort	Unclear: inhibits the reuptake of several neurotransmitters, including 5HT, NE, dopamine, and γ-aminobutyric acid	*Hypericum perforatum*	300-900

Clomipramine, nefazodone and venlafaxine are potent non-selective serotonin reuptake inhibitors. Citalopram S-enantiomer, escitalopram, is the most active isomer and is a more potent and more selective serotonin reuptake inhibitor than citalopram. Extracts of *Hypericum perforatum* (St. John's Wort) are used in many countries to treat depressive disorders. GABA: Gamma aminobutyric acid; NE: Norepinephrine; 5HT: 5-hydroxytryptamine.

main predictor of suicidal ideation. Other factors were lower age of disease onset, panic disorder, and social anxiety disorder[74]. Interestingly, these figures are not higher than those reported in non-parkinsonian patients with major depression. It is noteworthy to mention that active suicidal ideation, lifetime suicidal attempts are associated with early-onset depression and young age[75]. The lower prevalence of suicidal ideation in depressed PD patients might be due to the fact that the disease occurs in old age.

FACTORS ASSOCIATED WITH DEPRESSION

Factors associated with depression include increased severity of motor disability, greater impairment in

activities of daily living[28,34,36,39,43,76], and longer disease durations[25,33,31,39,42,61]. Depression is more frequent in the young onset PD[34,43,45,76-78]. Similarly, those with subthreshold depression are younger (approximately 5 years) than non-depressed patients[27]. In contrast, Riedel *et al*[39] found that depression rates were already substantially elevated at early PD stages and that depression was not linked with age, age at onset of PD, or disease duration. In their study, van der Hoek[42] observed no difference in the prevalence of depression among the motor subtypes of PD. The authors, however, noted a trend towards higher prevalence of depression in the tremor dominant group of patients. In contrast, Dewey *et al*[79] found that patients with right-sided onset of tremor had a lower risk of depressive symptoms compared with other presentations. Meanwhile, the side and type of initial motor symptoms were not related to the risk of later cognitive impairment.

Gender imbalance is common in depression in non-PD subjects. McKercher *et al*[80] reported a prevalence of major depression of 5.5% for men and 11.6% for women. The prevalence of atypical depression is also higher in women than in men (24.6% *vs* 17.3%)[81]. Studies also suggested that depressive symptoms were more likely to occur in females than in males[42,82,83]. Other researchers observed no significant difference in the prevalence of depression between men and women with PD[42].

Stressful life events have been implicated in the onset of episodes of major depression[58]. Stressful life events are independent predictors of depressive symptoms in older adults[84] and in those who experience depression recurrence, exposure to acute life events predicts the evolution of residual symptoms to recurrence[85]. Depression in PD is associated with a history of anxiety disorder and memory problems[34] and with dementia[39]. Having a history of depression prior to onset of PD was predictive of depression with PD[45]. Significantly more serious depression also occurs in subjects with a history of depression before PD compared with those without such history[86]. Rod *et al*[46] suggested an important role for life events in onset of depression in patients with PD. The authors found that more than 50% of their sample experienced major life events since diagnosed with PD with major depression occurring in 9.9%. It was also noted that each additional event was associated with a 56% higher risk of depression. These observations stress the importance of social support in the management of PD patients with depression. Stressful life events are also important in non-PD depressed subjects.

Anxiety, memory problems, hallucinations, sleep disturbances are more common in depressed PD patients compared with PD patients without depression[34,76]. Apathy, a possible feature of depression, can exist independently and is often associated with cognitive impairment[87]. Depression in PD is often associated with anxiety[28,34,41,50] and both depression and anxiety might be early symptoms during the prodromal phase of PD[88]. Anxiety and apathy are significant comorbid conditions of moderate and severe depression[89]. Anxiety coexisted with depression in 8.6%[66] or 41% of the PD patients[50]. The figures are not higher than those encountered in non-PD patients. Thus, in patients with current episode of depression, generalized anxiety disorder and panic disorder comorbidities were associated with unipolar depression in 37.1% and 31.4% of patients, respectively[90]. In late-life depression in non-PD subjects, the prevalence rate of comorbid anxiety disorders was 38.6%[91]. Brown *et al*[44] suggested the presence of two clinical phenotypes of depression in depressed PD subjects, "anxious-depressed" and "depressed", with a large proportion of patients have relatively isolated anxiety. Depression and anxiety disorders were often unrecognized and untreated and the comorbidity greatly exacerbated PD symptoms[92]. It is likely that anxiety and depression in PD are due to different pathophysiological mechanisms[41].

COURSE OF DEPRESSION AND THE EFFECT OF DOPAMINERGIC DRUGS

Symptoms of depression are among the most frequent non-motor symptoms in the premotor phase of the disease. de la Riva *et al*[9] reported that newly diagnosed, untreated patients with PD experienced more depression, fatigue, apathy, and anxiety than healthy controls all time points; these remained relatively stable in early disease. Depression and other neuropsychiatric symptoms appear to be amenable to antiparkinsonian drug therapy, suggesting that they are related to or part of the disease process. In this context, Nègre-Pagès *et al*[41] found that patients with depressive symptoms received more frequently levodopa and less frequently a dopamine agonist. Similar observations were reported by Hanganu *et al*[93] who found that higher levodopa (L-dopa) dosages correlated with worse depressive symptoms. In contrast, there was no significant correlation between dopamine agonists and worsening of depressive symptoms. Spalletta *et al*[10] found significant improvement over time in the depression severity (also memory performance, and motor symptoms) in newly diagnosed patients with PD after 6-12 mo of antiparkinsonian therapy. Kulisevsky *et al*[94] found that among neuropsychiatric symptoms in PD, only depression was influenced by the type of medication, being less prevalent following treatment with dopaminergic receptor agonists. This suggested that depression in these patients is related to the dopaminergic deficit. Other neuropsychiatric symptoms such as impulse control disorders and excessive daytime sleepiness, however, are increasingly associated with the use of these drugs[9]. Even *et al*[95] identified three possible subtypes of comorbid depression associated with PD. The first category of patients is those who would develop depression even if they had no PD

(nonspecific-casual comorbid dPD). The second subtype includes patients who would be depressed because of another disabling medical illness (nonspecific-reactive comorbid dPD). The third group of patients are those in whom depression is directly related to the underlying pathophysiology of PD (specific comorbid dPD). This latter subtype might be partly responsive to dopamine replacement, suggesting a role for other neurotransmitter systems in its pathogenesis. There are data, however, that suggest a negative impact of dopaminergic pharmacotherapy on cognitive function in depressed PD patients in contrast to non-depressed patients who performed better while on dopaminergic medication[96].

Some dopaminergic medications appear to have antidepressant action unrelated to their influences on motor function. Pramipexole is a non-ergot dopamine receptor agonist which has been shown to be effective in reducing unified Parkinson's Disease Rating Scale (UPDRS) in early PD and as an "add on" therapy in advanced disease[97,98]. The UPDRS is used to assess both motor and nonmotor symptoms by listing numerous items to be scored by the examiner[5]. Studies suggest that pramipexole possesses a direct antidepressant effect. Thus, Barone *et al*[99] compared pramipexole to sertraline in a randomized trial in PD patients with major depression but no motor complications. They found that both agents decreased depression scores throughout treatment. The proportion of patients who recovered was significantly higher in the pramipexole compared to the sertraline group (60.6% *vs* 27.3%). In an open study of pramipexole as an add-on to L-dopa therapy or single administration, the scores of depressive symptoms, UPDR Scale III, and freezing of gait improved. No correlation was observed between depression scores and motor functions, suggesting an antidepressant effect for pramipexole[100]. Barone *et al*[101] conducted a 14-wk randomized trial comparing pramipexole with placebo in patients with mild-to-moderate PD without motor fluctuations who had depressive symptoms. The authors found that pramipexole improved depressive symptoms. Selegiline and rasagiline are irreversible selective inhibitors of the enzyme MAO-B that are effective as an initial monotherapy in early PD and as adjunct therapy to L-dopa in advanced PD[102-104]. Frampton *et al*[105] tested the efficacy of selegiline transdermal application in a randomized, double-blind, multicentre studies in adult outpatients with major depressive disorder. They found that short-term treatment with selegiline (6-12 mg/d) was superior to placebo on most measures of antidepressant activity. Long-term treatment with a fixed dose of 6 mg/d selegiline was also superior to placebo as maintenance therapy. In addition to improving motor performance, treatment with rasagiline (2 mg/d) in newly diagnosed PD patients who also have comorbid untreated depression, has been shown to improve depression symptoms. Rasagiline appears to have direct antidepressant action since it especially improved symptoms uninfluenced by motor function such as mood, guilt, psychic anxiety, and hypochondria[106].

NEURO-IMAGING STUDIES

It has been suggested that the development of depression in PD is likely to represent an advanced and widespread neurodegeneration of both serotonergic and dopaminergic neurons[76]. Imaging studies suggested that brain dopamine deficiency might have a role in depression in PD patients. Studies with 18F-fluorodopa-PET in *de novo* unmedicated PD patients showed that higher depression scores were associated with lower striatal 18F-fluorodopa uptake, suggesting that impaired striatal dopaminergic function is related to depressive symptoms in these subjects[107]. Other studies using [(123) I] FP-CIT single photon emission computed tomography (SPECT) tracer binding to the dopamine transporter (DAT) reported significant decrease in DAT availability in patients with PD. There was an association between dopamine loss in the caudate nucleus (lower DAT binding) and depressive symptoms[108,109]. In one study, reduced DAT binding was reported in the striatum in the majority of patients with major depression, indicating a role for dopamine hypofunction in this disorder. A more pronounced decrease in DAT binding occurred in PD patients (SPECT imaging using 99mTc-TRODAT-1)[110]. Bui *et al*[111] suggested that the decrease in striatal uptake in the context of a depressive episode might be reversible. The authors observed improved PD symptoms and increased DAT uptake {[(123) I] FP-CIT SPECT} in a depressed PD patient following treatment with electroconvulsive therapy. Ceravolo *et al*[112], however, reported increased bilateral striatal (123) I-FP-CIT uptake (DAT density) associated with the severity of both depressive and anxious symptoms in newly diagnosed PD patients. This was attributed to a lack of compensatory mechanisms and that it might have a pathogenic role in affective symptoms by reducing the dopaminergic tone in the synaptic cleft.

Not only dopaminergic pathways are affected in PD, but also cholinergic, serotonergic, and noradrenergic ones[113,114]. The neurobiology of depressive disorders involves alterations in serotonergic, dopaminergic and noradrenergic neurotransmission[115,116]. This forms the basis for the use of drugs such as tricyclic antidepressants (TCAs), serotonin reuptake inhibitors, noradrenaline reuptake inhibitors in the pharmacological management of depressive disorders[117,118]. Politis *et al*[119] used ^{11}C-DASB PET, a selective *in vivo* marker of 5-HT transporter binding to assess serotonergic function in patients with PD. They found relatively higher ^{11}C-DASB binding in raphe nuclei and limbic structures in those with highest scores for depression symptoms which might reflect reduced extracellular serotonin levels and decreased serotonergic neurotransmission. Beucke *et al*[120] suggested that un-medicated PD

patients have a low serotonergic activity which might be related to the dopamine deficit. Thus, auditory evoked potentials (indicator of central serotonergic function) were decreased in patients with PD compared with healthy subjects, but this difference was abolished following L-dopa treatment for 12 wk. The authors also noted a trend towards a correlation between auditory evoked potentials and DAT of the unmedicated patients [using (123) I-FP-CIT SPECT].

NEED FOR ANTIDEPRESSANT DRUG THERAPY

The presence of depression in PD subjects is under-recognized and consequently untreated. For instance, Althaus *et al*[121] reported a prevalence of depressive symptoms in 35.4% of their sample. Antidepressant drugs, however, were prescribed in 25.0% of patients suffering from moderate to severe depression. Moreover, depression was largely undertreated because a significant proportion of patients continued to experience depressive symptoms despite antidepressant drug therapy. In another study minor and major depression were found in 36.3% and 12.9% of the subjects, respectively. Only 8.6% of the minor depressed patients and 30.3% of the major depressed patients were prescribed antidepressant drugs[42]. Moreover, de la Riva *et al*[9] found that approximately two-thirds of patients with PD who screened positive for depression were not taking an antidepressant.

The development of depression in subjects with PD has a major impact on the quality of life and activities of daily living. The presence of neuropsychiatric symptoms such as depression, apathy, sleep disturbance and anxiety is associated with more severe parkinsonism compared with patients without these symptoms[29]. Depression also impacts on other cognitive functions. In one study, significant subjective memory complaints were reported by approximately 15% of PD patients and these worsen with increasing severity of depressive symptoms[32]. Subjects with left hemibody onset of motor symptoms and depression exhibited worse working memory, greater disability and lower quality of life compared with those without depression (and also relative to depressed subjects with left hemibody onset of motor symptoms)[122]. It has also been suggested that the presence of depressive symptoms (as well as dopaminergic drugs, disease severity and the occurrence of cognitive impairment) might underlie the onset of psychotic type symptoms in the early stages of PD[123]. Successful treatment of depression leads to important, sustained improvements in the quality of life and disability in PD patients[124].

ANTIDEPRESSANT DRUGS

The TCAs and the monoamine oxidase inhibitors (MAOIs) were the first classes of drugs employed in the pharmacological management of depressive symptoms. These agents work by increasing the synaptic concentration of the monoamine neurotransmitters; norepinephrine (NE), serotonin [(5-hydroxytryptamine, (5HT)] and dopamine. The MAOIs inhibit the enzymatic metabolism of neurotransmitters. The TCAs inhibit the neuronal uptake of NE and 5HT. The TCAs dominated the pharmacological management of depressive disorders for more than 30 years. With the advent of the new generations of antidepressants such as the selective serotonin reuptake inhibitors (SSRIs), the serotonin and noradrenaline reuptake inhibitors (SNRIs) and the noradrenaline reuptake inhibitors (NRIs), TCAs are no longer considered first-line treatments[125-127]. Table 2 lists different classes of antidepressant drugs and their mechanism of action.

ANTIDEPRESSANTS USED IN PD

TCAs

These include the tertiary-amine tricyclics, such as clomipramine, imipramine, amitriptyline, doxepin and trimipramine, and the secondary amines, such as desipramine, protriptyline and nortriptyline. These drugs owe their antidepressant properties to inhibition of the neuronal uptake of the monoamine neurotransmitters; norepinephrine, and serotonin (5-hydroxytryptamine, 5HT). Individual agents differ in their relative potency to inhibit the reuptake of either NE or 5HT. The tertiary tricyclics, amitriptyline, imipramine, and clomipramine are more potent in blocking the serotonin transporter while the secondary tricyclics are much more potent in blocking the norepinephrine transporter. These drugs as well as the tetracyclic compounds maprotiline and amoxapine have been approved for use in major depression with the exception of clomipramine, which in the United States is approved for use only in obsessive-compulsive disorder[128,129]. The TCAs dominated the pharmacological management of depressive disorders for more than 30 years. With the advent of the new generations of antidepressants such as the SSRIs, SNRIs and the NRIs, TCAs are no longer considered first-line treatments[125-127]. The TCAs have the capacity to block alpha1-adrenergic, H1 histaminergic and muscarinic receptors. The side effects include anticholinergic effects such as dry mouth, blurred vision, urinary retention and constipation. Sedative and cognitive effects should make their use in the elderly be largely avoided. Their slowed clearance in old age leads to drug accumulation and increased frequency and severity of side effects[13,130]. TCAs also cause weight gain, and sexual dysfunction[131]. TCAs cause cardiac conduction defects and arrhythmias by blocking fast inward Na^+ channels on myocardial cells. Blockade of postsynaptic peripheral α-adrenergic receptors contributes to the postural hypotension associated with TCA use[132]. The use of antidepressants in general and in particular TCAs is associated with tachycardia[133].

TRICYCLIC ANTIDEPRESSANT DRUGS IN PARKINSON'S DISEASE

TCAs were the first class of antidepressant medications to be used for the treatment of depression in patients with PD. These agents were found more effective than placebo and even better than some of the SSRIs. Thus, Andersen *et al*[134] treated patients with nortriptyline for 16 wk and observed larger improvement compared with placebo. A meta-analysis by Frisina *et al*[135] of 24 placebo-controlled trials found that TCAs had a greater antidepressant effect relative to SSRIs and the mono-amine-oxidase inhibitor, selegiline. Side effect profile was, however, in favor of SSRIs. A more recent meta-analysis by Liu *et al*[136] concluded that TCAs might be the best choice when starting antidepressant treatment in patients of PD. In a study comparing amitriptyline and fluoxetine, Serrano-Dueñas[137] found that amitriptyline (approximately 35 mg/d) was better than fluoxetine at controlling the depression. Side effects that occurred in 15% of the patients on amitriptyline treatment, however, led these patients to abandon the drug. Antonini *et al*[138] compared low-dose amitriptyline (25 mg) to the SSRI sertraline (50 mg) on depression and quality of life in a prospective single-blind randomized study. Drugs were administered for 3 mo. Responder and completion rates were 83.3% and 75% for sertraline and 72.7% and 73% for amitriptyline, respectively. Sertraline and not amitriptyline treatment had a significant benefit on quality of life. (Whether the dopamine reuptake inhibition by sertraline is involved is an intriguing possibility!).

The short-term efficacy of desipramine was compared to that of citalopram, a SSRI in a double-blind, randomized, placebo-controlled study. The authors found that desipramine induced a more rapid improvement (after 14 d) in depression score than did an SSRI and placebo. After 30 d both drugs significantly improved depression. Thus a predominantly noradrenergic reuptake inhibitor TCA was faster than an SSRI in controlling depression in PD[139]. Moreover, nortriptyline, another TCA which mainly inhibits noradrenaline reuptake was found superior to placebo in decreasing depression scores. In this randomized, placebo controlled trial, the SSRI paroxetine controlled release (CR), however, was not efficacious. Response rates for nortriptyline, paroxetine CR, and placebo were 53%, 11%, and 24%, respectively[140].

TCAs possess antihistaminic effects which might be of benefit in those suffering from insomnia. One randomized pilot study assessed non-pharmacologic treatment or doxepin, compared to placebo in PD patients with insomnia. Compared to placebo, doxepin improved insomnia, sleep quality, clinical global impression of change. The drug also reduced fatigue severity and improved cognitive scores[141]. Clomipramine, a drug with prominent 5HT reuptake inhibitory action was reported to improve delusions and hallucinations in a parkinsonian patient with psychosis and comorbid depression[142].

Recently, a study by Paumier *et al*[143] in early PD patients showed that TCAs resulted in delaying the time to initiation of dopaminergic therapy compared with patients not on antidepressants. There were no changes in Unified PD Rating Scale (UPDRS) scores. The effect of TCAs thus could not be attributed to symptomatic effects.

Table 3 lists selected studies on the effect of TCAs on depressive symptoms in subjects with PD.

SSRIs

These agents are considered first line treatments of depression due to their more safety profile compared to the TCAs[144]. The SSRIs differ in their potency and selectivity in inhibiting serotonin reuptake and in their pharmacokinetics. The prototype SSRIs is fluoxetine which acts by blocking the reuptake of 5HT at the presynaptic neuronal membrane, thereby increasing its concentration in the synaptic cleft[145]. Fluoxetine has longer elimination half-life of 1-3 d after acute administration, while its active metabolite norfluoxetine has a half life of 7-15 d[128]. Its abrupt cessation is not likely to cause discontinuation reactions[146].

Fluvoxamine and paroxetine are other potent SSRIs with an elimination half-life of 15 and 21 h, respectively[144,147]. The abrupt discontinuation of paroxetine results in withdrawal symptoms, including nightmares, tremor, dizziness, insomnia, myalgias, and a "flu-like" syndrome[148]. It is thus advisable to taper the medication over several days, particularly in patients receiving more than 20 mg per day[145]. The drug is a first-line treatment option for major depressive disorder, dysthymia or minor depression[149].

The inability of citalopram to cause significant inhibition of hepatic enzymes made the agent an attractive agent for the treatment of depression, especially among the elderly and patients with comorbid illness requiring concomitant medicines[150,151]. Escitalopram is the pure S-enantiomer of the racemic compound citalopram and the pharmacologically active enantiomer of the racemate which have a more potent antidepressant than that of citalopram. Escitalopram is approximately 30-fold more potent than R-citalopram[152,153].

The SSRIs affect the reuptake of other neurotr-ansmitters. Thus, fluoxetine also acutely increases the extracellular concentrations of NE and dopamine (as well as 5HT) in prefrontal cortex[154] and unlike the other SSRIs possesses moderate affinity for the serotonin 2C receptor[152]. Paroxetine and sertraline possess moderate affinity for the human NE transporter and dopamine transporter, respectively[152]. Sertraline has been shown to increase extracellular levels of dopamine in the nucleus accumbens and striatum[155] which might have important clinical consequences. Paroxetine displays affinity for the muscarinic cholinergic receptor and causes a higher rate of anticholinergic effects, such as dry mouth, constipation, and cognitive

Table 3 Prevalence of depressive symptoms in subjects with Parkinson's disease in different studies

Stage of PD/type of patients	No. of patients/ sample size	Prevalence of depression/ depressive symptoms	Prevalence of other neuropsychatric symptoms	Ref.
Outpatients, non-fluctuating (21 de novo, 69 treated with levodopa or dopamine agonists)	90	Major depression in 21.1% (*vs* in 3.3% controls)	Panic disorders in 30% (*vs* 5.5% in controls) Dystimia in 18.8% (*vs* 4.4% in controls)	[26]
Outpatients with established PD	100	Major depression in 35%		[35]
Patients with PD presenting with non-motor symptoms. Retrospective study of pathologically-proven PD	91	Depression in 2.5%	Anxiety in 3.9%	[33]
Outpatients with established PD	50	Major depression in 42% (*vs* 10% of geriatric patients)		[28]
Nondemented patients with moderate to severe PD	111	Depression in 26.1% Subthreshold depression in 28.8%		[27]
Early untreated PD	175	Depression in 37%	Apathy in 27% Sleep disturbance in 18% Anxiety in 17%	[29]
New-onset PD patients	685	Depression in 72% (developed depression within ten years of symptomatic PD onset)		[36]
Outpatients with established PD	1086	Major depression in 15.6%		[37]
Outpatients with established PD	1449	Depression in 25%	Anxiety in 20% Dementia in 29% Psychotic syndromes in 12.7% Sleep disturbances in 49%	[38]
Outpatients with established PD	1449	Depression in 33.6%		[39]
Outpatients with established PD	150	Depression in 43% Depression without apathy in 13%	Apathy only in in 17% Apathy + depression in 43%	[40]
Non-demented PD subjects	105	38% borderline depression Major depression in 4.8%		[30]
Non-demented PD subjects	450	Depressive symptoms in 40% (*vs* 10% of controls)	Probable anxious signs in 51% (*vs* 29% of controls)	[41]
Patients with established PD	256	Minor depression in 36.3% Major depression in 12.9%		[42]
Patients with established PD	360	Depression in 41.3%	Only apathy in 23% Apathy + depression in 36.9%	[43]
Patients with established PD	202	Depression in 37.3%	Anxiety in 31.3%, Dementia in 25.3% Excessive daytime sleepiness in 59.4%	[31]
Patients with established PD	513	Depression in 8.6%	Anxiety alone in 22.0% Anxiety + depressive symptoms in 8.6%	[44]
Outpatients with established PD	158	Depression in 11% to 57% (depending on the definition of depression)		[45]
Outpatients with established PD	639	Depression in 66%		[34]
New-onset PD patients	221	Major depression in 9.9% (developed depression over 3-4 yr)		[46]
Outpatients with established PD	1449	Depression in 18.8%	Dementia in 13.9% had Dementia + depression in 14.3%	[47]
Non-demented PD subjects	95	Depression in 28%		[48]
Early stage PD	36	Depression in 36.1%	Anxiety in 27% Obsessive-compulsive symptoms in 52.8% Somatization in 66.7%	[49]
Outpatients with established PD	117	Depression in 56%	Anxiety in 55%	[50]
Patients with established PD (ambulatory and home residents)	886	Depression in 24.4%	28.4% dementia (20.6 % of ambulatory and 85.7 % of home residents)	[51]
PD patients with mild cognitive impairment	104	Depression in 40.4% (*vs* 16.6% in controls)	Subjective memory complaints 16.3% (*vs* 7.7% of controls)	[32]
Non-demented PD subjects	115	Major depression in 28.7% Subthreshold depression in 26.10%		[52]

PD: Parkinson's disease.

disruption, compared with other SSRIs. These effects may be particularly difficult to tolerate for elderly or concomitantly medically ill patients[156]. There are also data to suggest that long-term treatment with paroxetine increases GABA, glutamate, dopamine and noradrenaline levels in the brain[157].

The most common side effects associated with SSRIs include initial nervousness or agitation, anxiety, headache, insomnia, dizziness, dry mouth, gastrointestinal symptoms (nausea, diarrhea, constipation) and sexual dysfunction[144,158]. The use of SSRIs is likely to increase the risk of upper GI bleeding, and this effect is potentiated when these drugs are used in combination with nonsteroidal anti-inflammatory drugs or low-dose aspirin. Other antidepressant drugs did not appear to have an effect on the risk of upper GI bleeding[159]. Other studies reported increased risk of upper gastrointestinal bleeding after short-term SSRI use (7-28 d) intake in male but not female patients[160]. Prior use of SSRIs has also been implicated in increased stroke severity and mortality in patients with hemorrhagic stroke. This, however, was not seen in in SSRI users with ischemic stroke[161]. SSRI/SNRI antidepressants and in particular sertraline and escitalopram have been shown to increase the risk of hyponatraemia, especially in depressed patients aged > 63 years[162]. Recent evidence also implicates SSRIs with decreased bone mineral density and increased risk of hip fracture which appear to decline after discontinuation of these agents[163,164]. SSRIs are associated with a modest but statistically significant increase in the QTc interval with citalopram being associated with more QTc prolongation than most other SSRIs. The increase in QTc by TCAs is however, significantly greater than that of SSRIs[165].

SSRIs in PD

Case reports have associated some of the SSRIs with extrapyramidal side effects. Leo[166] in a review of case reports and case series of movement disorders attributed to SSRIs found that among the 71 cases reported in the literature, the most common side effect was akathisia, dystonia, parkinsonism, and tardive dyskinesia-like states, with a frequency of 45.1%, 292.%, 14.1% and 11.3% respectively. Fluoxetine was implicated in 74.6% of cases of SSRI-induced extrapyramidal symptoms. Other concomitant drugs that can contribute to the development of extrapyramidal symptoms were likely in 57.7% of reports. Caley *et al*[167] in a retrospective study of medical records of 23 outpatients with Parkinson's disease who were receiving or had received fluoxetine up to 40 mg/d, found that 20/23 of patients experienced no worsening of their symptoms.

Studies in PD patients with depression, however, have shown treatment with SSRIs to be mostly safe and efficacious. A study of 66 patients with non-fluctuating, depressed patients with PD found a significant improvement in depressive symptoms with citalopram, fluoxetine, fluvoxamine, and sertraline 6 months after starting treatment. There was no significant change in UPDRS scores. The study, however, comprised a small number of patients (15-16) in each drug subgroup[168]. Rampello *et al*[169] treated depressed (n = 16) and nondepressed (n = 14) PD patients with citalopram (up to 20 mg/d) and observed improved depressive symptoms in 15/16 patients with depression. Moreover, citalopram did not worsen motor performance and on the contrary improved bradykinesia and finger taps in subjects with and without depression on levodopa.

Studies have also indicated an ability of paroxetine to improve depression in depressed PD patients. Tesei *et al*[170] administered paroxetine (10-20 mg/d) to 65 outpatients with PD and depression and found improved depression scores in 52 patients after approximately 3 mo. Adverse reactions which occurred in 13 patients led them to stop treatment. There were also increased "off" time and tremor in 2 patients that reversed after stopping paroxetine. In another study by Ceravolo *et al*[171] 6 mo therapy of paroxetine (20 mg/d) improved depression without an effect on motor function (UPDRS scores). Reversible worsening of tremor was observed in one patient. Chung *et al*[172] who examined the motor effects of 2 wk of paroxetine and placebo on responses to 2-h levodopa infusions, found no effect for the drug on tapping scores or dyskinesia. Paroxetine increased baseline walking speed (prior to infusion) but with increased subjective perception of worsened balance. In a randomized, double-blind, placebo-controlled trial, both paroxetine and venlafaxine XR were efficacious in improving depression without effects on motor function. The mean 12-wk reductions in depression score were 6.2 points for paroxetine group and 4.2 points for venlafaxine XR[173]. A randomized, controlled trial of paroxetine CR, nortriptyline, and placebo in 52 patients with PD and depression, however, failed to demonstrate a benefit from paroxetine[142].

In their study, Kostić *et al*[174] administered fluoxetine at daily dose of 20 mg to patients with PD and mild depression. The authors reported significant improvement in depression and UPDRS scores. These correlated with steady state plasma concentrations of fluoxetine and its metabolite norfluoxetine. In an open label trial of 10 patients with PD and major depression, citalopram improved depression, anxiety and functional impairment significantly[175]. In another open-label study of 14 PD patients with major depression, escitalopram treatment was well tolerated with a significant decrease in depressive symptoms, although response rate was only 21%[176]. In a double-blind, randomized, placebo-controlled study of PD patients with major depression, citalopram (and desipramine) produced significant improvements in the depression score after 30 d[139].

Sertraline was found effective in relieving depression in patients with PD without significant effect on motor performance[177]. Sertraline was of comparable efficacy to amitriptyline in decreasing depression scores in PD patients with depression. In this prospective single-blind randomized study, the responder rates were 83.3% and 72.7% for sertraline and amitriptyline, respectively. Sertraline but not amitriptyline, improved quality of life (mobility, activities of daily living)[138]. Kulisevsky *et al*[178] in a large sample of 374 depressed PD patients of whom 310 completed the study found that treatment with sertraline decreased depressive scores and also improved UPDRS scores. There was

worsening of tremor in some patients. Sertraline in both the usual formulation and in the liquid oral concentrate was found efficacious in deceasing depressive scores. Quality of life improved with sertraline (clinical global impression-severity of illness scale and clinical global impression-global improvement scale scores) after 6 mo of treatment. This occurred without change in UPPDR Scale motor scores[179].

OTHER ANTIDEPRESSANTS

Trazodone and nefazodone

Trazodone and nefazodone are chemically related with complex serotonergic actions. These drugs antagonize 5HT2A and 2C postsynaptic receptors. Blockade of these receptors leads to facilitated neurotransmission through $5HT_{1A}$ receptors, which reduces anxiety levels. In addition both drugs inhibit the reuptake of 5HT to some extent. They thus possess antidepressant, and also some anxiolytic and hypnotic activity, and have favorable sleep architecture profile[180]. Nefazodone has weak affinity for cholinergic and noradrenaline α_1- adrenergic receptors and, therefore, is associated with less sedation and orthostatic hypotension than trazodone. The drug has favorable effect on sleep pattern in contrast to fluoxetine which has been shown to not improve sleep in depressed patients[181,182]. Thus, nefazodone would be suitable for depressed patients with prominent features of anxiety and agitation and loss of sleep[105]. Sedation, dry mouth, nausea, and dizziness are the more common adverse effects of nefazodone[183]. In the treatment of major depression, these agents do not differ from the SSRIs with respect to overall efficacy and tolerability[184].

In PD patients with depression, Avila *et al*[185] provided data suggestive of motor improvement (UPDRS score) after nefazodone, but not after fluoxetine treatment. Meanwhile, both drugs were equally effective as antidepressants. In another study, by Werneck *et al*[186] trazodone improved depression and motor function improved in the depressed patients treated with the drug.

Mirtazapine

Mirtazapine is a noradrenergic and specific serotonergic antidepressant. The drug increases noradrenergic and 5HT transmission *via* presynaptic α_2-antagonism. Mirtazapine increases the release of NE from central noradrenergic neurons by blocking the presynaptic inhibitory α_2-autoreceptors. It blocks the inhibitory α_2 heteroreceptors on serotonergic neurons, resulting in increased release of serotonin. Mirtazapine also blocks histamine H1 receptors, thus causing sedation, but has little effect on acetylcholine, dopamine or noradrenaline α_1 receptors. The most common side effects are dry mouth, sedation, increased appetite, and weight gain[144,187]. Mirtazapine has a faster onset of action compared with to SSRIs[188,189]. Case reports suggested a positive effect of mirtazapine on auditory[190] and visual[191] hallucinations in patients with PD and persistent psychosis without worsening motor symptoms. This antipsychotic effect of mirtazapine was attributed to 5HT-2A and/or 5HT-2C antagonism leading to dopamine release[190].

Venlafaxine

Venlafaxine is a serotonin and SNRI[144]. Venlafaxine has a rapid onset of clinical action (one week or two). In the treatment of in-patients with major depression venlafaxine was superior to fluoxetine[192]. It is used to treat melancholia (endogenous depression) and treatment-refractory depression[128]. Remission rates were significantly higher with venlafaxine than with an SSRI[193]. A single-blind study in elderly patients suffering from resistant major depression, found venlafaxine to be significantly superior to paroxetine in improving depression[194]. Adverse effects of the drug include nausea, somnolence, insomnia, and dizziness, constipation, sweating, nervousness, and abnormal ejaculation, cardiac conduction changes[128]. In non-fluctuating PD patients with depression, venlafaxine treatment for 8 wk improved depression without changes in UPDRS scores[195]. In a randomized, double-blind, placebo-controlled trial in depressed PD patients, venlafaxine extended release was effective in improving depression. The mean 12-wk reductions in depression score were 6.2 points for paroxetine group and 4.2 points for venlafaxine extended release[173].

Atomoxetine

In subjects with PD and depression, treatment with the SNRI atomoxetine was not found efficacious in relieving depressive symptoms. Global cognitive performance and daytime sleepiness, however, significantly improved[196].

Duloxetine

Duloxetine is a serotonin and noradrenaline reuptake inhibitor. In an open-label study in PD patients with major depression, duloxetine 60 mg once daily significantly improved depression scores and activities of daily living without worsening rigidity or tremor[197].

Table 4 lists selected studies on the effect of SSRIs and other antidepressant drugs on depressive symptoms in subjects with PD.

NON-PHARMACOLOGICAL TREATMENT MODALITIES

Neurostimulation

Electrical neurostimulation techniques include deep brain stimulation (DBS) of subthalamic motor nuclei or globus pallidus internus, transcranial magnetic stimulation (TMS), and electroconvulsive therapy.

Table 4 Studies on the effect of antidepressant drugs on depressive symptoms in Parkinson's disease subjects with depression

Drug	Study design	Sample size	Study objectives	Outcomes	Adverse effects	Ref.
Fluoxetine		23	Effects of fluoxetine (up to 40 mg/d) on motor performance	20/23 patients experienced no worsening of parkinsonism		[167]
Fluoxetine, fluvoxamine, citalopram, and sertraline	Open-label prospective study	62 depressed patients with PD (without dementia or motor fluctuation) (15 patients received citalopram, 16 fluoxetine, 16 fluvoxamine, and 15 sertraline)	Effects of SSRIs on motor performance and depressive symptoms	↓↑ UPDRS scores Significant improvements in depression with all SSRIs		[168]
Fluoxetine/ amitriptyline	Randomized study	77 patients with PD (37 received fluoxetine and 40 received amitriptyline)	Comparing fluoxetine (20-40 mg/d) and amitriptyline (25-75 mg/d) at low doses on depressive symptoms	Amitriptyline better controlled depression at 3, 6, 9 and 12 mo, respectively	15% abandoned amitriptyline because of side effects	[137]
Fluoxetine	Prospective, controlled, open-label study	18 patients with PD and mild depression without dementia	Influence of fluoxetine (20 mg/d) on motor functions	Significant improvements in scores of depression and Parkinson's disability		[174]
Paroxetine			To assess the tolerability of paroxetine (20 mg once per day)	Improved depression UPDRS scores ↓↑	Reversible worsening of tremor in one patient	[171]
Paroxetine		65 outpatients with PD and depression	To assess the tolerability of paroxetine (10-20 mg once per day)	Improved depression	20% stopped paroxetine because of adverse reactions Increased "off" time and tremor in 2 patients (reversible)	[170]
Paroxetine CR/ nortriptyline	Randomized, placebo controlled trial	52 patients with PD and depression	To evaluate the efficacy of paroxetine CR and nortriptyline in treating depression	Nortriptyline was superior to placebo for the change in depressive scores Paroxetine CR was not		[140]
Paroxetine/ venlafaxine	Randomized, double-blind, placebo-controlled trial	115 subjects with PD	To compare efficacy and safety paroxetine and venlafaxine extended release in treating depression in PD	Both paroxetine and venlafaxine XR significantly improved depression UPDRS scores ↓↑		[173]
Citalopram		46 non-demented patients with PD. 18 depressed and 28 non-depressed	Effect of citalopram on motor and nonmotor symptoms of depressed and nondepressed patients with IPD	Improvement in mood in 15/16 patients Motor performance ↓↑ Improved bradykinesia and finger taps in patients with and without depression		[169]
Citalopram	Prospective, open label trial	10 patients with PD and major depression, without dementia	Effects of citalopram on depressive symptoms	Significant improvement in depression and in anxiety symptoms and functional impairment		[175]
Escitalopram	Open-label study	14 Parkinson's disease patients with major depression	Effects of escitalopram on depressive symptoms	↓ in depressive symptomatology score (response and remission rates were only 21% and 14%)		[176]
Sertraline	Open-label pilot study	15 patients with PD and depression	To evaluate the safety and efficacy of sertraline to treat depression in PD	Significant improvement in depression UPDRS scores ↓↑	Side effects in 1/3 2 patients discontinued sertraline	[177]
Sertraline		54 PD patients with depressive disorders	Comparing efficacy of sertraline in the usual formulation and in the liquid oral concentrate	Improved depression on both formulations Improved clinical global impression-severity of illness scale		[179]
Sertraline		374 PD patients with depressive symptoms	Long-term effects of sertraline on motor status	Improved UPDRS ↓ Anxiety ↓ Depression	8% discontinued medication for adverse events (gastrointestinal) Worsening of tremor in some patients	[178]

Sertraline/ amitriptyline	Prospective single-blind randomized study	31 patients with PD and depression	Assessment of the effect of sertraline (50 mg) or low-dose amitriptyline (25 mg) on depression and quality of life	↓ Depression by both drugs Sertraline improved quality of life ↓↑ UPDRS scores		[138]
Sertraline/ pramipexole	Randomized trial	67 outpatients with PD and major depression but no motor fluctuations and/or dyskinesia	To compare pramipexole with sertraline	Both sertraline and pramipexole improved depression Pramipexole caused more recovery compared to sertraline (60.6% *vs* 27.3%) Pramipexole improved UPDRS motor subscore	14.7% withdrew from the sertraline group	[99]
Nefazodone/ fluoxetine	A pilot randomized trial	Depressed patients with PD	To assess the effect of nefazodone on extrapyramidal symptoms in depressed PD patients	Nefazodone significantly improved UPDRS score Both nefazodone and fluoxetine were equally effective in treating depression		[185]
Trazodone	Randomized trial	20 PD patients with and without depression	To test the ability of trazodone to improve depression and motor function	Significantly improved depression Improves motor function in depressed patients		[186]
Venlafaxine	Prospective study	14 non-fluctuating PD patients with depression	To investigate the therapeutic efficacy of venlafaxine	Improved depression scores UPDRS scores ↓↑		[195]
Atomoxetine, a SNRI	Randomized placebo- controlled study	55 subjects with PD depression atomoxetine or placebo	To assess efficacy of atomoxetine (80 mg/d) in treating depression	Failed to improved depression Improved global cognition Improved daytime sleepiness		[196]
Duloxetine	Non-comparative, open-label, multi-center study	151 patients	To evaluate the tolerability, safety, and efficacy of duloxetine 60 mg once daily in PD patients with major depressive disorder	Improved depressive scores Improved activities of daily living Tremor ↓↑ Rigidity ↓↑	8.6% discontinued the study due to side effects	[197]

PD: Parkinson's disease; SNRI: Selective norepinephrine reuptake inhibitor; UPDRS: Unified Parkinson's disease Rating Scale.

DBS surgery

This involves inserting microelectrodes into the basal ganglia nuclei, *e.g.*, subthalamic nucleus or globus pallidus internus. In advanced stage PD, deep brain stimulation of subthalamic nucleus improves motor function, motor fluctuations, dyskinesia, activities of daily living, quality of life and allows dopaminergic treatment reduction or withdrawal in a subset of patients[198-206]. Improvement in anxiety, depression, and fatigue has also been reported following subthalamic stimulation[199,200,202,206-208]. In addition, patients with severe PD subjected to bilateral subthalamic nucleus DBS were reported to have had significantly longer survival[209]. The effect of subthalamic DBS on depression, however, might not be maintained. In one study, motor UPDRS-III scores decreased within 18 mo postoperatively compared with preoperative and the medication control group. Self-Rating Depression Scale and Hamilton Rating Scale for Depression decreased within 6 and 3 mo postoperatively, respectively[202]. Cognitive deterioration[203,207,210], decline in verbal fluency and in abstract reasoning, episodic memory and executive function[211], depression[202,205,212,213], apathy[212], worsening of apathy[214] as well as the unmasking of previous psychiatric problems[215] might complicate the procedure. The increase in affective-cognitive symptoms of depression after DBS might reduce the procedure-induced motor improvement[213]. Suicide has also been reported among patients undergoing subthalamic nucleus DBS, despite clear motor improvements[213,216]. Other studies, however, reported no increased risk for suicide ideation and behaviors among PD patients subjected to subthalamic nucleus or globus pallidus interna DBS surgery[217]. Operative complications include infection, intracerebral hematoma, chronic subdural hematoma, electrode fracture, and incorrect lead placement, phlebitis, and pulmonary embolism[204,205,218].

TMS

Brain stimulation with TMS is a noninvasive approach of electrically stimulating neurons in the human cerebral cortex that is capable of modifying neuronal activity both locally and at distant sites[219]. The technique of TMS involves the passage of an electrical current through a copper-wire coil placed on the scalp. A brief, rapid time changing magnetic field is created at the level of the

coil which then induces a small electrical current in the underlying brain. Depolarization of neuronal membranes and generation of action potentials follows. In repetitive TMS (rTMS), repeated electrical pulses are generated in the cortex[220,221]. Repetitive TMS of the left dorsolateral prefrontal cortex was approved for the treatment of major depression in United States in 2008[222]. The technique appears to be without side effects[222,223]. Maruo *et al*[223] observed that three consecutive days of HF-rTMS over the M1 foot area in patients with PD failed to improve depression and apathy scales, despite significant improvement in UPDRS-III compared to sham stimulation.

Electroconvulsive therapy

In PD patients with refractory psychiatric symptoms, electroconvulsive therapy (ECT) led to improvement in symptoms of psychosis and motor symptoms with no adverse effects[224]. There are case reports that ECT was successful in the treatment of severe anxiety[225], and obsessive compulsive disorder[226] in PD, depression and parkinsonism in drug-induced parkinsonism[227]. Usui *et al*[228] reported improvement of psychosis and severity of PD in eight patients with levodopa or dopamine agonist-induced psychosis. The technique has also been used to treat depression in patients implanted with DBS. Chou *et al*[229] found that ECT dramatically improved major depression with psychotic features that occurred after bilateral subthalamic nucleus DBS surgery. Nasr *et al*[230] reported the treatment of severe depression with psychotic features and the decline in physical and mental states using ECT in a patient implanted with DBS. A randomized, double-blind trial of transcranial electrostimulation in early PD, however, found no significant effect on anxiety or depression and also on motor symptoms[231].

Neurosurgical ablation techniques

Surgery, *e.g.*, pallidotomy, subthalamotomy are sometimes used to alleviate motor symptoms in advanced PD patients refractory to medical treatment[232]. Surgery still might be resorted to in some instances where DBS is contraindicated or following complications necessitating removal of the implanted device[233]. In one study, lasting improvement in depression and apathy and no cognitive deterioration were reported in patients with advanced PD subjected to simultaneous bilateral subthalamotomies[234]. In another study, advanced PD subjects with depressed mood subjected to left-posteroventral pallidotomy performed worse on measures of verbal list learning and story recall when compared to non-depressed subjects or right-posteroventral pallidotomy subjects with depressed mood[235].

Cognitive behavior therapy

This psychotherapeutic treatment option improves comorbid depression and anxiety and the quality of life in PD patients[236,237]. In one study, patients on cognitive behavior therapy reported greater reductions in depression scores, anxiety, and improved quality of life compared with the clinical monitoring group[238]. Cognitive behavior therapy delivered *via* telephone to persons with PD also proved useful in improving psychiatric symptoms[239].

Insight from animal studies

The use of animal models of PD has greatly enabled our understanding of the pathogenetic mechanisms of depression in PD and also helped to identify potential therapeutic targets. Many studies employed the neurotoxin 6-hydroxydopamine (6-OHDA) which when injected into the striatum of rats induces marked depletion of dopamine, serotonin and also noradrenaline in the striatum[240]. Using this model, Tadaiesky *et al*[241] demonstrated that anxiogenic- and depressive-like behaviors occur early in the course of experimental parkinsonism analogous to that in early phases of human PD. This occurred along with alterations of dopamine, serotonin and noradrenaline in the striatum. Mild anxiogenic effects were also reported in 6-OHDA-lesioned rats. These effects were not amenable to treatment with L-dopa[242]. Branchi *et al*[243] found depressive-like behavior but reduced anxiety and a marked change in social behavior and no learning or memory difficulties in 6-OHDA-lesioned rats. Courtière *et al*[244] using the reaction time task test provided data that 6-OHDA lesioned rats had cognitive impairment similar to PD patients. These studies indicated that behavioral changes also occur in early phases of experimental PD. Studies in rodents also allowed the evaluation of such techniques as, TMS, rTMS and DBS. Thus high-frequency electrical stimulation of the subthalamic nucleus in rats was shown to increase striatal dopamine efflux, thereby indicating that the benefit from this technique is probably due to enhanced dopamine release within the basal ganglia[245]. Extracellular levels of 5HT in both striatum and medial prefrontal cortex also decreased following high-frequency electrical stimulation of the subthalamic nucleus even in dopamine-denervated rats. Changes in 5HT neurotransmission might therefore account for the depression seen in some patients following DBS of the subthalamic nucleus[246]. Ghiglieri *et al*[247] found that rTMS increases striatal excitability and rescues corticostriatal long-term depression in experimental parkinsonism in rats.

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MINIREVIEWS

Rituximab in neuromyelitis optica: A review of literature

Ericka Wong, Vijay A Vishwanath, Ilya Kister

Ericka Wong, Vijay A Vishwanath, Ilya Kister, Department of Neurology, NYU Multiple Sclerosis Care Center, New York, NY 10026, United States

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Correspondence to: Dr. Ilya Kister, MD, Department of Neurology, NYU Multiple Sclerosis Care Center, 240 E 38th St, New York, NY 10026, United States. ilya.kister@nyumc.org
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Abstract

Neuromyelitis optica spectrum disorders, or neuromyelitis optica (NMO), is an autoimmune disease of the central nervous system that must be distinguished from multiple sclerosis. Therapeutic approaches to relapse prevention in NMO include immunosuppressants and monoclonal antibodies. Rituximab, a monoclonal antibody that targets CD20 antigen expressed on the surface of pre-B, mature B-lymphocytes and a small subset of T-lymphocytes, has been widely used for the treatment of NMO. In this review, we aim to summarize global experience with rituximab in NMO. We identified 13 observational studies that involved a total of 209 NMO patients treated with rituximab. Majority of rituximab-treated patients evidenced stabilization or improvements in their disability scores compared to pre-treatment period and 66% of patients remained relapse-free during treatment period. Monitoring rituximab treatment response with CD19+ or CD27+ cell counts appears to improve treatment outcomes. We offer clinical pointers on rituximab use for NMO based on the literature and authors' experience, and pose questions that would need to be addressed in future studies.

Key words: Neuromyelitis optica; Rituximab; Longitudinally extensive transverse myelitis; Optic neuritis; CD19+; CD27+

Core tip: Relapsing neuromyelitis optica (NMO) is an autoimmune disorder of the central nervous system that often results in severe disability and death if untreated. Rituximab, an anti-CD20 monoclonal antibody, appears to be a promising treatment option for NMO. In this review, we summarize the results of 13 observational studies that assessed efficacy of Rituximab in neuromyelitis optica. On average, 66% of patients remained relapse-free during treatment period and in the majority of patients disability scores have stabilized or improved. Monitoring response to rituximab with CD19+ and CD 27+ cell counts appears to improve outcomes.

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INTRODUCTION

Neuromyelitis optica spectrum disorders, hereafter

referred to as "Neuromyelitis optica (NMO)", is an autoimmune disease of the central nervous system (CNS)[1]. Diagnostic criteria for NMO have undergone several revisions in recent years, but core clinical syndromes - longitudinally extensive transverse myelitis and optic neuritis, have been retained. The most recent iteration of the diagnostic criteria for NMO is based on International Panel for NMO Diagnosis consensus paper[2]. In Aquaporin-4-IgG (AQP4-IgG) seropositive patients, diagnosis can be made after a single NMO-compatible relapse. AQP4-IgG seronegative NMO criteria include evidence of dissemination in space as well as at least one well-recognized syndrome of NMO, such as ON, LETM or intractable vomiting/hiccups[2].

NMO has been reported worldwide with prevalence ranging from 0.52-4.4/100000[3]. In Western counties, NMO is rare relative to multiple sclerosis (MS) - ratio of 1:50-100[4-6], but in the developing counties, NMO may constitute up to 40% of all CNS autoimmune diseases[7]. Prognosis and treatment in NMO and MS are different. Five-year mortality of untreated relapsing NMO was 68% - a much higher rate than in MS - and half of the surviving patients had permanent monoplegia or paraplegia[8]. Disease modifying therapies for relapsing MS, such as Interferon and the remarkably effective Natalizumab, fail to prevent, and may even precipitate, relapses of NMO[9,10]. Current strategies for relapse prevention in NMO include immunosuppressants and monoclonal antibodies[11,12], but efficacy of these approaches has not been tested in randomized clinical trials. One of the most promising agents for NMO is rituximab (RTX), a monoclonal antibody that targets CD20 antigen expressed on the surface of pre-B, mature B-lymphocytes (but not normal plasma cells)[13] and a small subset of T-lymphocytes[14]. Our review aims to summarize global experience with RTX for the treatment of NMO and offer clinical pointers based on the literature and authors' experience.

RATIONALE FOR B-CELL DEPLETING THERAPY IN NEUROMYELITIS OPTICA

Landmark pathologic study by Lucchinetti *et al*[15] concluded that "the pronounced Ig reactivity co-localizing with complement activation at sites of vessel damage may be due to a specific antibody targeted to a vascular antigen"[15]. This prediction was borne out two years later when Lennon *et al*[16] discovered an exquisitely NMO-specific autoantibody directed against AQP-4, a water channel found in astrocytic end-feet[16]. Current conceptualization of NMO pathogenesis is that anti-AQP4 auto-antibody binds to AQP-4[17] and initiates complement-mediated astrocyte injury and inflammatory reaction that secondarily affects oligodendrocytes and leads to demyelination and neuronal loss[18]. This hypothesis successfully explains many features of NMO, but does not account for the diversity of observed pathologic findings[19], nor for disease pathogenesis in anti-AQP4- Ab seronegative NMO patients, who comprise approximately 30% of NMO cases in the United States[5].

In view of the central role of humoral autoimmunity to NMO pathogenesis, it is not surprising that B cell lineage depletion would be proposed as a rational therapeutic strategy. Indeed, shortly after discovery of anti-AQP-4 Ab, Cree *et al*[20] reported an open label study of RTX in NMO that demonstrated high efficacy of the drug in all but one of their patients. A number of reports on RTX efficacy in NMO from different parts of the globe have since appeared. This review included all English-language studies that involved 5 or more RTX-treated NMO patients and recorded either relapse rate/number before and after treatment with RTX, or expanded disability status scale (EDSS) scores before and after treatment with RTX, or both outcome measures. We searched PubMed for "Neuromyelitis Optica" and "Rituximab" and cross-checked references. We identified 25 articles and finally 13 articles were included in this study, which met our inclusion criteria. Two articles were excluded for multiple treatments used; four were excluded for other diseases included; four were excluded for having less than 5 patients; and another two articles were excluded for not documenting treatment effect. Two unpublished case series of RTX-treated NMO that were presented at recent international neurologic conferences were also included in this review; additional data was obtained from the authors[21,22].

EFFICACY OF RTX IN NMO

Thirteen studies met our inclusion criteria. The total number of treated patients was 209, of whom the overwhelming majority were women (approximately 90%). Table 1 summarizes demographic and clinical data from the 13 studies. Four out of the thirteen studies reported median annualized relapse rate (ARR) before and after RTX[20,23-25]. Median ARR prior to treatment ranged from 1.7-2.6 and it decreased to 0-0.4 during the treatment period, which was usually 1-2 years. Two studies reported change in mean ARR[26,27], which decreased from 1.2-2.4 pre-treatment to 0-0.3 after treatment was started. The remaining seven studies specified total number of relapses before and after RTX as detailed in Table 1[21-22,28-32]. In 11 out of 13 studies, 48%-75% of patients were relapse-free during treatment period. There were two exceptions: in the study by Lindsey *et al*[32] only 3 out of 9 patients (33%) were relapse-free; this study was critiqued for possible under-dosing of RTX[33]. In the study of Yang *et al*[28] none of the 5 patients experienced any further relapses while on RTX.

In all but one study, some patients "failed to respond" to treatment. Javed *et al*[22] characterized nearly 33% of their NMO patients as "non-responders" based on the fact that RTX failed to delay further relapses, which occurred within 2.5 mo post treatment[22] (Table 1). Phenomenon of disease rebound in the immediate post-

Table 1 Case series of rituximab in neuromyelitis optica

Ref.	Country; Type of study	No. of patients (n = 209)	Mean age at RTX; % Female	% Anti-AQP4 Ab seropositive	RTX Protocol /treatment duration	ARR before RTX	ARR after RTX	% Relapse-free	EDSS (median) before --> after RTX
Cree *et al*[20]	United States; Retrospective	8	37[1]; 88%	N/A	A- treatment B- retreatment	2.6 (median)	0 (median)	75% (6/8 pts at 12 mo f/u)	7.5--> 5.5
Jacob *et al*[23]	United States/ England; Retrospective	25	43[1]; 88%	70%	A or B; median interval between cycles-8 mo 19 mo follow up	1.7 (median)	0 (median)	72% (17/25 at 12 mo estimated)	7--> 5 2 patients deceased
Bomprezzi *et al*[31]	United States; Retrospective	18	46 (+/-12); 83%	67%	B	15 pts-RTX tx and 7 had relapses. 42% (5/12) showed "positive treatment effects", the other 7 continued to relapse despite RTX therapy		53% (8/15)	Severe disability from NMO' - 10 patients
Bedi *et al*[24]	United States; Retrospective	23	46[1]; 91%	72%	A or B; 32.5 mo	1.87 (median)	0 (median)	74 % (17/23 pts)	7--> 5.5
Pellkofer *et al*[30]	Germany; Prospective	10	47[1]; 90%	100%	B; number of cycles of RTX 1-5	Ever before RTX: 1.3 mo, 12 m before RTX: 2.4 mo, 24 m before RTX: 1.72 mo, With RTX: 0.93 mo		50% (5/10 at 12 mo estimated)	6--> 6.5[1] 1 patient deceased
Javed *et al*[22]	United States; Retrospective	15	34; N/A	N/A	B; patients were given RTX 1g x1 usually 6-9 mo after the initial dose	2/10 had 2 relapses in 6 mo post RX. 5 non-responders had mean of 1.45 (median 1) relapses in mean 12.2 (median 10) mo		67% (RTX delayed further relapses for 9 mo or more)	N/A
Gredler *et al*[26]	Austria; Retrospective	6	38; 83%	66%	375 mg/m^2; no of infusions 3-16 (mean = 6.67), interval between infusions 3.3-11 mo	2.5 (mean)[1]	0.4 (mean)[1]	67% (4/6)	5.25--> 2.25[1]
Ip *et al*[25]	China; Prospective	7	52; 85%	66%	A or B: Mean # trx courses: 2.85. median 2	Mean ARR = 2.4 median ARR = 2[1] 5 became relapse free. 2 had 50% reduction over median 24 mo		71 % (5/7)	8--> 7
Lindsey *et al*[32]	United States; Retrospective	9	N/A; 89%	60%	A or B: Mean duration: 74.2 mo	3 pts with early relapses in first month after RTX, 4 pts (including 1 pt with early relapse) with later relapses		33% (3/9)	3.5--> 4.3[1]
Kim *et al*[27]	South Korea; Retrospective	30	38.4 (± 10.5); 90%	77%	A or B; mean 61 mo (range 49-82 mo), median 60 mo	2.4 (mean)	0.3 (mean)	70% (21/30 at 2 yr f/u)	4--> 3
Yang *et al*[28]	China; Prospective	5	42[1]; N/A	80%	100 mg (50-59 mg/m^2) RTX IV 1 dose/wk for 3 cons wk; mean duration: 12.2 mo	1.16[1] (mean)	0[1] (mean)	100%	4.5--> 4
Mealy *et al*[29]	United States; Retrospective	30	45[1]; 83%	50%	B; median of 20 mo (range 5-83 mo)	Total pretreatment ARR- 2.89	Total post-treatment ARR- 0.33	67% (20/30)	N/A
Farber *et al*[21]	United States; Retrospective	23	38; 100%	74%	Mean of 22 mo (range 2-96 mo)		Median ARR was 0.24; mean was 1.02 (SD = 1.36)	48% (11/23)	N/A

[1]Estimated based on results table or manuscript when possible. A or B (in RTX protocol column): There were two treatment protocols used- Protocol A with 4 doses RTX 375 mg/m^2 IV wk for 4 wk; Protocol B with 2 doses of RTX 1000 mg IV 2 wk apart. NMO: Neuromyelitis optica; ARR: Annual relapse rate; EDSS: Expanded disability status scale; RTX: Rituximab; AQP4: Ab - aquaporin 4 antibody; N/A: Not available.

induction period was documented by Perumal *et al*[34] in 6 out of their 17 patients; however most patients with

post-induction relapses evidenced disease stabilization with further RTX dosing and so need not be necessarily classified as "true non-responders". Perumal *et al*[34] hypothesized that cytokine release and increases in BAFF and AQP4 levels that immediately follow RTX infusion[35] may precipitate a post-infusion relapse in highly active NMO patients.

Continual disease activity can occur in RTX-treated NMO patients with complete depletion of B cells[29,30,32,36]. Risk factors that would predict non-responsiveness to RTX are presently unknown. It was suggested that RTX non-responders may require not only B-lymphocyte elimination with RTX, but an additional, "broad-spectrum immune-suppressant" to achieve disease remssion[31]. This strategy has been successfully adopted in the treatment of Rheumatoid Arthritis, where RTX is often combined with Methotrexate or Cyclophosphamide[13]. We discuss potential mechanisms that may explain lack of response to RTX in "Variability in responses to RTX treatment" section below.

EDSS scores before and after RTX were reported in 9 out of 13 studies. In 7 of the 9 studies, EDSS at last follow-up was lower than prior to RTX initiation. Exceptions were the studies by Lindsey *et al*[32] and Pellkofer *et al*[30], in which EDSS at last follow-up increased by 0.8 and 0.5, respectively, compared to pre-treatment EDSS[30,32].

ADVERSE EVENTS

Two studies recorded fatal outcomes in RTX-treated NMO patients. In the study of Jacob *et al*[23], one patient died from a brainstem NMO relapse and another succumbed to suspected septicemia. Pellkofer *et al*[30] reported one death due to presumed cardiovascular failure that occurred 3 d after a rituximab infusion.

Adverse events were not systematically documented across the studies, so estimates of their prevalence are not possible. A number of infections have been observed - mostly, herpetic rashes and tuberculosis reactivation. RTX treatment carries a small risk of progressive multifocal leukoencephalopathy (PML) - 1 case per 25000 individuals in one large cohort of patients with rheumatoid arthritis[37]. No cases of PML in RTX-treated NMO patients have been reported to date, though there was a single case report of PML in NMO patients treated with azathioprine[38]. Overall, adverse events profile of RTX in NMO appears to be consistent with known safety profile of the drug[13]. Infusion reaction to RTX are very common, but can usually be mitigated by pre-treatment with intravenous steroids anti-histamine and slow titration of RTX.

DOSING OF RTX AND BIOMARKERS OF TREATMENT RESPONSE

The majority of studies used one of two "induction protocols": 375 mg/m^2 IV once a week for four consecutive weeks ("protocol A" in Table 1), or 1000 mg IV infused two weeks apart ("protocol B"). Timing of subsequent doses either followed a fixed schedule - with typical time to the next infusion cycle of 6-9 mo or was based on monitoring parameters. The most commonly used test for monitoring B cell suppression was CD19+ count assessed by flow cytometry. Since RTX interferes with the direct analysis of CD20 cell surface antigen *via* flow cytometry due to its mechanism of action, CD19+ antigen, which is largely co-expressed with CD20, is used as a surrogate marker to assess extent of B cell depletion[26]. However, CD19+ count may also overestimate degree of B cell depletion[39]. RTX typically depletes CD 19+ counts to undetectable levels (< 10 cells per μL) within 2-4 wk of infusion[13].

Table 2 summarizes the use of biomarkers to monitor treatment response to RTX in NMO. Several studies showed that CD19+ B cell population greater than 1% of lymphocyte total is a risk factor of a relapse. Farber *et al*[21] measured CD19 counts post-relapse and during periods of stability, and noted higher B cell counts in the immediate post-relapse period. Yang *et al*[28] suppressed CD19+ count to less than 1% in all their patients and were able to achieved complete eradication of relapses, despite lower doses of RTX used[27]. Pellkofer *et al*[30] used monthly, highly sensitive flow cytometry measurements to demonstrate that complete B cell suppression led to sustained clinical stabilization in most patients. Bomprezzi *et al*[31] showed that B cells become undetectable within 2 wk of the first dose of RTX, but rise to 2%-12% at the time of a relapse[31]. The early rise in CD19+ cells correlated with radiologically proven relapses, and 5 out of 7 patients experienced a relapse when CD19+ B cell population exceeded the 1% threshold[31]. In summary, preponderance of evidence favors suppressing CD19+ B cell to ≤ 1% of the total lymphocyte count in NMO patients for maximal efficacy.

Efficacy of "low dose" RTX on CD19 counts was assessed in two studies. Yang *et al*[28] used RTX 100 mg infusion once a week for 3 consecutive weeks, which was followed by the next RTX 100 mg dose when CD19+ cells were > 1% and the memory CD19+ CD27+ B cells were > 0.05%. In this regimen, CD19+ cells started to increase in 4 of the 5 patients approximately 140 d after the initial RTX infusion, necessitating a re-infusion[28]. In the Greenberg *et al*[40] study, RTX 100 mg dose resulted in early re-population of B cells compared to the 1000 mg dose[40]. The median number of days for CD19 population to reach threshold of 2% was 133 d in the 100-mg per dose arm *vs* 259 d in the 1000-mg per dose arm[40].

Kim *et al*[27] proposed that CD27$^+$ memory B cells may be a more relevant biomarker of pathogenic B cells depletion in NMO than CD19+ B cells[26]. Memory B cells can elicit larger and faster responses to antigen than naive B cells, and so may be more relevant to disease pathogenesis. Re-emergence of CD27+ memory B cells above the therapeutic target (< 0.05% of PBMCs) may occur even when CD19+ B cells levels were < 0.5% of

Table 2 Monitoring parameters in Neuromyelitis optica patients treated with rituximab

Ref.	Monitoring parameter/comments
Cree *et al*[20]	CD19 levels- when detectable, patients were re-treated. CD 19 followed bimonthly. 2 protocols-planned infusions every 6 mo or 12 mo
Jacob *et al*[23]	CD19 not routinely monitored. Some RTX given when B-cell counts detectable either 6 or 12 mo in intervals or when CD19+ became detectable
Bomprezzi *et al*[31]	Flow cytometry used to test circulating B cells. Suggest clinical relapses occurring while on RTX therapy correlate with reconstitution of circulating B cells. Correlated that even early rise in CD20+ cells correlated with radiologically proven relapses. B cells had re-sent between 2% and 12% at time of new attack. Total of 7 patients relapsed after RTX-5 had acute event when B cell counts just returned to greater than 1%, whereas 2 patients continued to relapse despite B cells being undetectable. Detected significant variability in timing of reconstitution of normal values, which implies that scheduling of doses of RTX can be adjusted accordingly
Bedi *et al*[24]	CD19 cell counts planned every 2-3 mo, but not collected systematically for report
Pellkofer *et al*[30]	Measured lymphocyte subsets by flow cytometry; B cell depletion defined as counts below 0.01×10^9 /L. B cells became undetectable in 9 out of 10 patients within 14 d after 1st dose. Time of B-cell repopulation varied. After 3 patients experienced a relapse shortly after reappearance of B cells, RTX given at fixed interval every 6 to 9 mo, which this led to improved outcomes
Javed *et al*[22]	"Non-responders" were defined as clinical attack < 6 mo post rituximab treatment, when B cell count was still undetectable
Gredler *et al*[26]	Flow cytometry used; B cells quantified using following combinations of monoclonal antibodies: CD3/19/45, 19/27/45, 19/38/45. Two patients out of 6 had relapses while B-cells were absent
Lindsey *et al*[32]	4 patients had relapses after more than 1 mo when peripheral B cell count "very low". Case 1: CD19 increased to 250 cells/μL had sensory relapse, no further symptoms for 18 mo; Case 2: Had relapses with CD19 count of 0; Case 3, 4, 6 no further relapses; Case 5: CD19 1 cells/μL at 10 mo, 12 cells/μL at 13 mo and subsequent relapses; Case 7--continued to have relapses with 1 cell/μL at 7 mo, 4 cells/μL at 12 mo. Case 8: CD19 count 3 cells/μL, with continued relapses; Case 9: continued relapses with CD19 1 cells/μL
Kim *et al*[27]	Blood samples obtained every 6 wk in 1st year, every 8 wk in second year. Therapeutic target for CD 27+ memory B cell depletion was less than 0.05% of PBMCs. Patients received additional infusion of 375 mg/m^2 if frequency of re-emerging memory CD27+ B cells in PBMCs exceeded 0.1% by flow cytometry. CD 19 B cells counts measure- less than 0.01×10^9 /L or less than 0.5% of PBMCs (considered B cell depletion in prior studies. 60%-65% relapses occurred when CD19 were depleted. Authors argue CD27+ more informative biomarker than CD19
Yang *et al*[28]	Goal of CD19+ B cells to less than or equal to 1%, as well as CD19 CD27 B cells to less than or equal to 0.05% of PBMCs. All with no relapses despite low doses of RTX (100 mg single infusion and follow up infusion at mean of 35 wk)
Mealy *et al*[29]	CD19 cell counts tested monthly, repeated dosing scheduled on detection of CD19 greater than 1% of total lymphocyte population or at regular 6 mo intervals
Farber *et al*[21]	Total of 23 relapses, of which 70% occurred when B cells < 1% of lymphocytes. 7 relapses (30%) occurred when B cells greater or equal to 1% of lymphocytes. CD19 > 1% was associated with higher rate of relapses

RTX: Rituximab.

PBMCs. Perhaps, presence of memory cells in patients with ostensible absence of CD19+ cells can be explained by a recent study in which loss of CD19 surface antigen from healthy donor B cells exposed to rituximab *in vitro* was not necessarily associated with B cell death[41]. In the study of Kim *et al*[27], no relapses were observed in 29 out of 30 patients in whom CD27+ memory B cell fraction was below the therapeutic target. This important finding is corroborated by the small series of Yang *et al*[28], cited above. More studies are needed to determine if CD27+ should replace CD19+ as the biomarker of choice in monitoring response to RTX in NMO.

Pellkofer *et al*[30] studied utility of AQP4-Ab, total B cell counts and B cell fostering cytokines, such as BAFF (B cell activating factor) or APRIL (a proliferation-inducing ligand) as biomarkers in NMO. They found that disease activity correlated with B cell depletion, but not with AQP4-Ab or APRIL levels[30]. Relationship between CD19 counts and Anti-AQP4 titers was analyzed by Jarius *et al*[17], who concluded that administration of RTX was followed by a "prompt and marked decline" in AQP-4 Ab, though the auto-antibody remained detectable in nearly all patients[17].

VARIABILITY IN RESPONSES TO RTX TREATMENT

The mechanisms responsible for variability in RTX treatment responses are unclear. An early, post-infusion relapse may be related to incomplete elimination of pathologic B cells as well as transient increase in B cell activating factor, anti-AQP4 Ab titers and other cytokines following infusion[34]. Greenberg *et al*[40] reported an NMO patient who was "resistant" to RTX: after an initial fall of CD19 cell count to 0, patient continued to experience clinical relapses and marked early return of B cells at 91 d after RTX, which could not be suppressed by further doses of RTX. This appears to be an exceptional case, as most "true" non-responders in the Greenberg *et al*[40] study - 6 out of 8 - had CD19 count below 2% at the time of relapse. Kim *et al*[27] also noted that 13 out of 20 relapses (65%) occurred even when CD19 B cell fraction was less than 0.5% PBMCs[27]. Lindsey *et al*[32] ask whether in patients with continued relapses despite complete B cell suppression, pathogenic T-cell may play a relatively more prominent role in pathogenesis. It is also possible RTX does not completely eliminate pathogenic clones in the periphery

or that CD19+ count overestimates the degree of peripheral B cells depletion[41]. Furthermore, peripherally administered monoclonal antibodies have limited penetration across the blood-brain barrier - typically CSF concentration is < 0.1% of serum antibody concentration[42]. Although CSF Rituximab concentration may be considerably higher if blood-brain barrier is perturbed[39], its concentration may still be insufficient for elimination of B cells from CSF.

CONCLUSIONS AND QUESTIONS FOR FUTURE STUDIES

Thirteen observational studies from across the world (Table 1) have documented stabilization or improvement in disability scores in a majority of NMO patients upon initiation of RTX. Pooling data across the studies shows that 66% of patients were relapse-free throughout the treatment period (typically 1-2 years). It is possible that with a more rigorous monitoring of response with CD19+ or CD27+ biomarkers and improved strategies to avoid relapses in the post-induction period even more impressive results could be achieved. Although the studies in our review have been uncontrolled and mostly retrospective and so subject to various biases (*e.g.*, ascertainment bias, selection bias, publication bias), they are consistent in demonstrating robust treatment response. Considering the natural history of untreated NMO[8], it would seem highly unlikely that the observed reduction of relapses and improved disability scores in RTX-treated patients is accounted for solely by artifacts of data collection or regression to the mean. The accumulated weight of evidence, in authors' opinion, casts doubt on the possibility of genuine clinical equipoise in NMO at the present time.

Important questions remain with regard to place of RTX in the treatment algorithm of NMO. A recent retrospective review concluded that RTX had the lowest failure rate compared to the commonly used oral immunosuppressants[5]. Based on this data, and overall efficacy of RTX in the published studies, RTX should be strongly considered in any NMO patient who continues to relapse on oral immunosuppressants[13]. The question of whether RTX should replace prior treatment or be combined with it remains unresolved. Combination therapies, in wide use for rheumatologic diseases, have not received sufficient attention in neuro-immunology and will need to be studied more in the future. An acute treatment often used during NMO relapse is plasma exchange (PLEX)[43]. Efficacy and safety of PLEX in other refractory systemic autoimmune disease have been shown in several studies[44,45] and its role as maintenance therapy of NMO is currently being investigated[46].

Should RTX be the agent of choice for all previously untreated patients with NMO? The authors would consider RTX as a first-line therapy in a patient with aggressive disease course as well as in the older NMO patients, who tend to have worse outcomes[47]. It is less clear whether risk-to-benefit ratio calculation would favor RTX in milder and earlier cases, *e.g.*, in an AQP4-Ab seropositive patient after single relapse.

What would be the optimal timing for initiating RTX? If, as suggested by some studies[22,32,34], RTX could exacerbate NMO in the immediate post-infusion period, it would probably be safer to initiate RTX after a period of stability rather than during an acute exacerbation. This question requires further study as the data is conflicting. When switching a patient to RTX, a prudent recommendation is to avoid discontinuing prior therapy prematurely, as delay in starting RTX could put the patient at risk of relapse[24]. In our practice, we routinely continue treatment with an oral immunosuppressant for at least one month after RTX is started. With regard to timing of repeat RTX cycles, the literature supports use of CD19+ and, possibly, CD27+ cell count, to monitor treatment response (Table 2). One goal of treatment should be to keep these counts below threshold levels.

Important questions remain regarding long term safety and efficacy of RTX, and duration of therapy. There is little data with regard to long-term safety of RTX in NMO, but the long-term safety record of RTX in rheumatoid arthritis is reassuring[48]. Would it be safe, from NMO standpoint, to discontinue treatment with RTX after a (prolonged) period of stability? A recent study documented that a period of several years of no disease activity after RTX is discontinued is possible in some patients, though 2 out of 4 patients in that series experienced relapses after years of quiescence[49]. Considering the potentially devastating consequences of NMO relapse, routine discontinuation of RTX and "watchful waiting" is probably not advisable.

It is hoped that randomized clinical trials, several of which are under way now (*e.g.*,[50]) as well as multi-center collaborative observational studies based on NMO registries, such as online NMOBase registry (www.msbase.org), could provide data on long-term safety and efficacy of RTX in NMO and help resolve the unanswered questions raised in our review. Quality of observational studies in NMO could be improved by adherence to accepted guidelines[51], especially with respect to reporting outcomes (relapse rates and disability scores).

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MINIREVIEWS

Resolution of idiopathic intracranial hypertension after sustained lowering of cerebrospinal fluid pressure

Peter C Gates

Peter C Gates, Deakin University, Neurology Department, Barwon Health, University Hospital, Geelong VIC 3220, Australia
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Correspondence to: Peter C Gates, Professor, Deakin University, Neurology Department, Barwon Health, University Hospital, PO Box 281, Geelong VIC 3220, Australia. prof.petergates@gmail.com
Telephone: +61-3-42150711
Fax: +61-3-42150757
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Abstract

Idiopathic intracranial hypertension (IIH) is a syndrome of headache due to raised intracranial pressure (ICP) where the cerebrospinal fluid (CSF) is normal and there is no alternative pathology on imaging. The aetiology is unknown. This review questions many of the prevailing views regarding aetiology and treatment of IIH. It explores the concept that there is a vicious cycle of fluctuating raised ICP leading to secondary compression of the transverse sinuses and further elevation of ICP. It also raises the question as to whether this vicious cycle could be relieved by prolonged drainage of CSF as seen in Lumbar puncture induced low-pressure headache or alternatively a lumbar drain.

Key words: Lumbar puncture; Cerebrospinal fluid drainage; Idiopathic intracranial hypertension; Low-pressure headache

Core tip: Resolution of idiopathic intracranial hypertension can be achieved by prolonged cerebrospinal fluid drainage as seen with Lumbar puncture induced low-pressure headache.

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INTRODUCTION

Idiopathic intracranial hypertension (IIH) also referred to as benign intracranial hypertension or pseudotumour cerebri is a syndrome of headache due to raised intracranial pressure (> 20 cm H_2O in non-obese patients and > 25 cm H_2O in obese patients) where the cerebrospinal fluid (CSF) is normal and there is no alternative pathology on imaging. Patients present with headache, with or without visual obscuration's and or various cranial nerve palsies most commonly a 6th nerve palsy. In the early stages visual acuity is normal, the blind spot is enlarged and virtually all patients have bilateral papilloedema, although rare cases have been reported where papilloedema is absent[1-3]. The major consequence of untreated or undertreated IIH is visual

loss.

AETIOLOGY OF IIH

The aetiology is unknown with many suggested causes[4-6] most of which to this author do not make sense.

To this author there is only one constant feature present in all patients with IIH and that is the presence of fluctuating raised intracranial pressure. Intracranial pressure (ICP) monitoring has demonstrated the presence of B-waves (rhythmic oscillations occurring every 1-2 min. The ICP rises in a crescendo manner to levels 20–30 mmHg higher than baseline and then falls abruptly)[7,8]. These B-waves almost certainly explain the fluctuations of CSF pressure measurements seen with a Lumbar puncture (LP) and I would like to suggest the intermittent compression of the transverse venous sinuses that King and his colleagues elegantly demonstrated is secondary to and not the primary cause of the raised ICP[9].

What triggers the initial elevation of CSF pressure is unclear. The majority (but not all) patients are obese and females of child bearing age (there are occasional reports of males affected by IIH[10]). It is feasible that the demonstrated elevation of right atrial pressure in obese individuals transmitted to the cerebral venous system[11] could be the very initial trigger[12]. If this is the mechanism then the obvious question is why does this not occur in obese males and why do non obese females develop IIH? The answer is unknown; could it be that female hormones present in premenopausal women reduce the stiffness of the venous walls and predispose them to compression? The author has not been able to find any literature to support such a hypothesis.

The fact that obesity is not present in every patient would indicate that it is not the primary cause of IIH, but increases the risk of developing this condition. There are many other suggested causes of IIH[13,14], the fact that they are not present in every patient must raise significant doubt about their primary role in the aetiology of this condition.

An identical clinical picture is seen in some patients with cerebral vein thrombosis, leading to the suggestion that obstructed venous drainage may play a role in IIH. In recent years stenting of transverse sinuses has also led to a resolution of IIH[15-19], despite the fact that it is well established that the narrowing in the transverse sinuses is secondary to the raised pressure[9]. There is one simple clinical observation that rules out transverse sinus narrowing as the cause of IIH, it not present in every patient and it is not a constant feature in patients in whom it is seen. If the transverse sinus narrowing is secondary to the raised pressure then the question is how does stenting lead to a resolution of IIH in some cases? Pickard *et al*[20] have suggested on the basis of CSF and venous sinus pressure measurements that, in many cases of IIH, there is functional obstruction of venous outflow through the dural sinuses. Raised pressure of CSF (Pcsf) partly obstructs venous sinus outflow, thereby increasing sagittal sinus pressure (pss) which, in turn, leads to a further rise in Pcsf, et sequor. They further suggest that this vicious cycle can be interrupted by draining CSF. Is it possible that stenting helps break this cycle of fluctuating ICP and intermittent venous compression, allowing venous drainage and subsequent normalisation of ICP? Supporting this concept is the observation by McGonigal *et al*[21] of resolution of IIH and transverse sinus stenosis in a patient following a lumbo-peritoneal shunt where the patient developed a low-pressure headache.

There is no consensus about what is causing the raised pressure. Logically the pressure within a closed space reflects the contents of the space and the rigidity of the wall. The skull is rigid, the ventricles are slit like, there is no collection of CSF over the hemispheres, the venous sinuses are compressed and arterial pressure is raised secondarily to the raised ICP. It therefore stands to reason that there must be increased fluid in the cerebrum.

The concept that the raised intracranial pressure is related to fluid in the interstitial space is supported by recent studies on CSF dynamics. The traditional view that the majority of CSF is produced by the choroid plexus, circulates through the ventricles and the subarachnoid space to be absorbed by the arachnoid villi has recently been challenged[22]. The CSF circulation also comprises a pulsatile to and fro movement throughout the entire brain with local fluid exchange between blood, interstitial fluid and CSF[23,24]. There is a growing consensus that the interstitial fluid and CSF are mainly formed and reabsorbed across the walls of CNS blood capillaries with aquaporins playing a role[25]. It is now believed that there is a continuous bi-directional fluid exchange at the blood brain barrier and the cell membranes at the border between CSF and the interstitial fluid spaces[24].

The total volume of CSF is estimated to be 150 mL in adults with 25 mL in the ventricles. It has also been estimated that nearly 30% of CSF production may come from the ependyma[23]. CSF production is estimated to be 0.37 mL/min or approximately 500-600 mL/d[24]. If IIH represents a vicious cycle of raised intracranial pressure due to an increase in interstitial fluid with secondary venous compression this could explain why stenting could break the cycle by abolishing the venous compression. It might also explain the observation that an uncomplicated LP would not break the cycle as CSF would re-accumulate rapidly. It could also explain how a low CSF pressure that would occur with a continuous leak of CSF in the setting of a low-pressure headache could also break the cycle. The CSF must be leaking at a greater rate than it is being produced.

TREATMENT OF IIH

There are many review articles[4,26,27] that discuss the current treatment if the initial LP fails to lead to a

resolution, treatment options include serial LP's, medical therapy (weight loss, Acetazolamide, Topiramate or Octreotide) and surgical intervention (bariatric surgery, lumbo-peritoneal drain, transverse sinus stenting or optic nerve fenestration). As Batra and Sinclair comment "the aetiology is poorly understood and there are no evidence-based guidelines on the management of the disease".

It is not the intention of this article to discuss these various treatment options in detail suffice to say that medical therapy tends to be employed for mild cases of IIH and surgical intervention for the more severe cases with a tendency to favour optic nerve fenestration if vision is threatened. Rather I would like to explore the concept of "resetting the abnormally elevated pressure to normal" by prolonged CSF drainage reflecting our own observations[28-30] and a review of the literature.

LP is used to confirm the diagnosis and occasionally a single LP[31,32] or several LP's[33] can result in resolution of IIH but how this occurs is unclear. In the study of children by Weisberg *et al*[33] 20 to 50 mL of CSF was removed each time but they did not comment on the closing pressure or the whether patients developed low-pressure headache. When serial LP's are employed the CSF pressure is reduced to a normal level, 10-20 cm H_2O. We would argue that CSF is replaced very rapidly and unless the pressure is lowered to below normal and or the CSF is drained at a rate higher than it is replaced the vicious cycle of elevated intracranial pressure cannot reverse.

In refractory cases a lumboperitoneal shunt is often recommended. These run the risk of infection and recurrence of IIH when they become occluded. The CSF is often shunted to maintain the CSF at a normal pressure and to avoid a low-pressure headache from over drainage. One case in this series developed a low pressure headache after insertion of a lumboperitoneal shunt requiring removal, following which she experienced a resolution of IIH. We suspect the reason the IIH "recurs" with blockage of the shunt is that it was never reversed in the 1st case by the shunt, but rather the CSF pressure was maintained at a level that leads to a resolution of headache and papilloedema but not low enough to reverse the excess fluid in the intracellular space.

In 2009 we observed a young non obese female with IIH who had bilateral narrowing of the transverse sinuses demonstrated on magnetic resonance venography (MRV). MRV (using the same methodology) immediately before and 15 min after the next two LPs showed partial resolution of the transverse sinus narrowing when the pressure was reduced from 50 cm H_2O to 11cm H_2O and complete resolution of the bilateral transverse sinus narrowing when the pressure was reduced from 47 cm H_2O to 8 cm H_2O[28]. On the basis that stenting can lead to a resolution of IIH, we postulated that lowering the CSF pressure lower than usually recommended, could result in a resolution of the transverse sinus narrowing and therefore a resolution of IIH. This proved not to be the case, in several patients where the CSF pressure was reduced to less than 10 cm H_2O (a level where we had demonstrated resolution of the transverse sinus narrowing, see above) the IIH persisted[30].

At about the same time we observed a patient with IIH who had developed a low-pressure headache after an LP. Our initial reaction was to suspect the original diagnosis of IIH was incorrect, how could a patient with markedly elevated pressure develop a low-pressure headache it did not seem to make sense. On review of the patient's medical record it was clear that she fulfilled the diagnostic criteria[34-37].

A low-pressure headache represents prolonged drainage of CSF resulting in a low pressure, usually less than 5 cm H_2O. Clinically the headache of IIH is distinct from that of low-pressure headache with the latter abolished by lying flat with the foot of the bed elevated, but the only way to differentiate with certainty between IIH and low-pressure headache is to measure the CSF opening pressure. We subsequently undertook a review of all cases of IIH seen at the Geelong hospital. One patient had developed a low-pressure headache after the insertion of a lumbo-peritoneal shunt that had to be removed. IIH resolved in this patient but recurred some years later. A second patient developed a low-pressure headache in the setting of a temporary lumbar drain with permanent resolution of IIH. There were 10 other patients who had complete resolution of IIH (average follow-up 3 years (range 3 mo-10 years) following the development of a low-pressure headache. In 2 patients low CSF pressure was confirmed by LP (5 and 7 cm H_2O). There was one patient in whom the IIH persisted and who was clinically suspected of developing a low-pressure headache; the low pressure was not confirmed by LP in this patient[29,30]. One young non obese female patient has subsequently relapsed.

One possible explanation for these observations is that a low-pressure headache represents prolonged drainage of CSF reducing the external pressure on the transverse sinuses, relieving the physiological stenosis of these sinuses and allowing the vicious cycle of raised pressure to normalise. If this interpretation is correct then a way to explore this concept is to undertake a study of patients with IIH using controlled lumbar drainage, a technique that has been employed to control medically refractory increased intracranial pressure[38]. The CSF may need to be drained at a faster rate than has been traditionally recommended and the duration of drainage is uncertain. This could be explored in a multicentre study where the rate of and the duration of CSF drainage could be varied with each centre learning from prior experience. After the period of drainage the CSF pressure could be measured after a period of clamping of the drain for a minimum of 2 h (enough time for the volume of CSF in the ventricles to be restored, *i.e.,* 25 mL with a production rate of 0.37 mL/min). If the CSF remains elevated then one could undertake a further period of drainage at a faster

rate and/or for a longer period, once again measuring the CSF pressure after clamping the drain. An online database could be established to share observations and hopefully establish the ideal rate and duration to drain the CSF.

CONCLUSION

This paper has explored the concept that IIH may represent a vicious cycle of elevated intracranial pressure, triggered by unknown factor(s) that could potentially be interrupted by prolonged drainage of CSF as seen with a post-LP induced low-pressure headache or by prolonged lumbar drainage without the necessity of inserting a permanent lumbo-peritoneal shunt or transverse sinus stenting. The lumbar drain would need to drain CSF at a faster rate than 0.37 mL per minute in order to reduce the CSF pressure to a level low enough for the increased interstitial fluid to diminish back to its normal state.

Until such an approach is confirmed patients with severe IIH particularly if vision is threatened should be managed along conventional lines.

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MINIREVIEWS

Involvement of leak K^+ channels in neurological disorders

Hiroki Toyoda

Hiroki Toyoda, Department of Neuroscience and Oral Physiology, Osaka University Graduate School of Dentistry, Osaka 565-0871, Japan
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Correspondence to: Hiroki Toyoda, PhD, Department of Neuroscience and Oral Physiology, Osaka University Graduate School of Dentistry, 1-8, Yamadaoka, Suita, Osaka 565-0871, Japan. toyoda@dent.osaka-u.ac.jp
Telephone: +81-6-68792884
Fax: +81-6-68792885
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Abstract

TWIK-related acid-sensitive K^+ (TASK) channels give rise to leak K^+ currents which influence the resting membrane potential and input resistance. The wide expression of TASK1 and TASK3 channels in the central nervous system suggests that these channels are critically involved in neurological disorders. It has become apparent in the past decade that TASK channels play critical roles for the development of various neurological disorders. In this review, I describe evidence for their roles in ischemia, epilepsy, learning/memory/cognition and apoptosis.

Key words: Leak K^+ channel; TWIK-related acid-sensitive K^+ channel; Neurological disorder

Core tip: The leak K^+ conductance generated by TWIK-related acid-sensitive K^+ (TASK) channels is crucial for neuronal excitability. Because of the substantial expression of TASK channels in the brain, it is possible that these channels are responsible for numerous neurological disorders. However, little is known about the roles of TASK channels in the development of neurological disorders. In this review, I introduce the molecular basis of leak K^+ channels and describe the possible roles for TASK channels in several neurological disorders.

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INTRODUCTION

The neurological disorders are diseases of the brain, spinal cord, and nerves that make up the nervous system. There are a large number of neurological disorders such as epilepsy, Parkinson's disease and stroke. To date, many studies have been demonstrated that various ion channels expressed in the nervous system are involved in the development of neurological diseases[1,2]. The ion channels are classified into voltage-gated, ligand-gated, mechanosensitive and leak channels based on the control mechanism, while being classified into Na^+, K^+, Ca^{2+} and Cl^- channels based on the ion selectivity[3]. In recent years, the molecular basis of ion channels has been elucidated through the development of the molecular cell biology and genetic engineering method. However, much of the roles of ion channels in pathophysiological conditions including neurological

disorders remains unclear[4]. In this review, I will discuss the roles of leak K^+ channels in neurological disorders. In particular, I will focus on the TWIK-related acid-sensitive K^+ (TASK); TWIK, for tandem P domains in a weak inwardly rectifying K^+) channels (*e.g.,* TASK1 and TASK3) due to the high expressions in the central nervous system.

LEAK K^+ CHANNELS

Based on the structural features, K^+ channels are classified into three major families[5]. Members of the first family of K^+ channels include the voltage-gated K^+ channels (the delayed rectifier and transient voltage-dependent K^+ channels)[6] and Ca^{2+}-dependent K^+ channels[7] and form tetramers with each subunit containing six transmembrane domains and one pore domain. Members of the second family of K^+ channels include the inwardly-rectifying K^+ channels such as ATP-sensitive K^+ channels[8] and form tetramers with each subunit containing two transmembrane domains and one pore domain. Members of the third family of K^+ channels include the leak K^+ (two-pore-domain K^+) channels[5,9] and form dimers with each subunit containing four transmembrane domains and two pore domains. In excitable cells such as neurons, a negative membrane potential is critical for electrical signaling, and it has long been considered that this key mechanism is largely mediated by leak K^+ currents. However, the molecular basis for characterizing functional properties of leak K^+ currents remained unknown until recently. In the 1990s, the discovery of the *KCNK* gene family has been described whose members generate the hallmark properties of leak K^+ currents[9]. In mammalians, fifteen subunits have been identified and divided into six subfamilies (TWIK, TREK, TASK, TALK, THIK, and TRESK) on the basis of sequence similarity and functional resemblance[4,9]. The TWIK group includes the weakly inwardly rectifying channels (TWIK1, TWIK2, and the nonfunctional KCNK7); the THIK group includes halothane-inhibited THIK1 channel and related non-functional THIK2; the TREK group includes the arachidonic acid and mechanosensitive channels (TREK1, TREK2, and TRAAK); the TALK group includes the alkaline-activated channels (TASK2, TALK1, and TALK2/TASK4); the TASK group includes acid-sensitive channels (TASK1, TASK3, and the nonfunctional TASK5); the TRESK group includes Ca^{2+}-activated channels (TRESK1). Among fifteen subunits of leak K^+ channels described above, TASK1 and TASK3 are widely expressed in the central nervous system[10].

TASK CHANNELS

TASK channels are two-pore–domain channels that generate pH-sensitive K^+ currents with little time-dependence and weak rectification[5]. In heterologous expression systems, TASK5 was found to be inactive while TASK1 and TASK3 were able to form functional homomeric channels[9]. In addition, there is evidence that TASK1 and TASK3 might form functional heterodimers *in vitro* and *in vivo*[11,12]. The unitary conductance of TASK3 channel (approximately 28 pS) is about two times larger than that of TASK1 channel (approximately 14 pS)[13]. Although the macroscopic currents arising from these two channels are similar, the sensitivity to extracellular pH is different. The pK for TASK1 inhibition is approximately 7.4 while that for TASK3 is approximately 6.7[13]. TASK channels are inhibited by extracellular acidification, local anesthetics and G-protein-coupled receptors[5]. In contrast, TASK channels are activated by phospholipids and volatile anesthetics such as halothane and isoflurane[5].

INVOLVEMENT OF TASK CHANNELS IN NEUROLOGICAL DISORDERS

Ischemia

Neuronal damage caused by ischemic stroke is a major health care problem for persistent disability and death in clinical practice[14]. When ischemic state occurs, the transient membrane depolarization is induced in neurons. Consequently, the release of neurotransmitters such as glutamate, neuropeptide and Zn^{2+} is enhanced[15]. It is well known that the excessive glutamate causes neurotoxicity including neuronal dysfunction and degeneration. When the ischemic events continue to occur, cell death is induced[16]. On the other hand, mild hypoxia can induce neuroprotective signaling cascades that prevent neuronal death[17,18]. The activation of K^+ channels causes membrane hyperpolarization, which increases cell survival during cellular stress conditions. The decreased neuronal activity and the resultant lower metabolic demands could enhance neuronal survival under stress conditions. Thus, the protective effect of K^+ channels would reduce the development of ischemic stroke.

TASK1 and TASK3 channels are sensitive to acidic pH and hypoxic conditions. In addition, TASK3 homomeric channels are selectively suppressed by Zn^{2+}[19]. Considering that acidic pH and hypoxia are observed and the release of Zn^+ is enhanced during ischemic conditions, it is likely that TASK1 and TASK3 channels are involved in the development of ischemic stroke. Indeed, the roles of these channels in the ischemic stroke development have been revealed by pharmacological inhibitors and genetic knockout (KO) mice. In a study using a mouse model of cerebral ischemia, transient middle cerebral artery occlusion (MCAO), the infarct volume in TASK1 KO mice was significantly larger than that in its control mice while there was no significant difference in the infarct volume between TASK3 KO and its control mice[20]. The increased infarct volume could be mimicked by the TASK1 inhibitor anandamide[20]. Furthermore, in a study using a mouse model of permanent MCAO, the expression of TASK1 channel gene reduced the infarct volume, most likely

by a general influence on blood pressure[21]. These finding suggest TASK1 expressed in the brain decreases neuronal damage when stroke occurs.

Epilepsy

Epilepsy is a brain disorder that is characterized by the presence of spontaneous episodes of neuronal discharges[22]. Excessive and/or synchronous discharges in the brain cause the disruption of consciousness and disturbance of sensation and movement[22]. K^+ channels contribute to nearly all aspects of cellular electrical signaling and are important determinants of seizure susceptibility[23]. Therefore, K^+ channels have been considered as practical targets for anti-epileptic drug development.

In pathological conditions such as ischemia and epilepsy, it has been demonstrated that the extracellular pH was changed in the brain[24,25]. In the CA1 hippocampal areas, recurrent epileptiform activity caused biphasic pH shifts, consisting of an initial extracellular alkalinization followed by a slower acidification[25]. The authors indicated that the different extracellular pH shifts between CA1 and dentate gyrus might have caused the regional difference in seizure susceptibility between these two areas[25]. Because TASK channels are highly sensitive to changes in extracellular pH, several studies implicated the involvements of these channels in the generation of epilepsy. The changes in neuronal excitability within the hippocampus are one of the hallmarks of temporal lobe epilepsy[26]. Therefore, it is conceivable that TASK channels expressed in the hippocampus play essential roles in the generation of epilepsy. First, the role of TASK1 channels in epilepsy was investigated in the hippocampus of gerbils[27]. Between the hippocampi of young seizure-resistant (SR) and seizure-sensitive (SS) gerbils (1 to 2 mo old), there was no difference in the TASK1 and TASK2 immunoreactivites. In adult SS gerbil hippocampus, TASK1 immunoreactivity in astrocytes was higher compared to the adult SR gerbil hippocampus. After seizure events, TASK1 immunoreactivity was significantly downregulated in astrocytes of the SS gerbil hippocampus. Furthermore, several anti-epileptic drugs selectively reduced the TASK1 immunoreactivity in astrocytes of the SS gerbil hippocampus[27]. These findings indicated that upregulation of TASK1 channels in astrocytes may be responsible for the seizure activity of adult SS gerbils and that downregulation of TASK1 channels in astrocytes may suppress the seizure activity. In addition to TASK1 channels, the role of TASK2 channels in epilepsy was examined by using a rat model of experimental temporal lobe epilepsy[28]. Following status epilepticus, TASK2 expression in the CA1 pyramidal cell layer was downregulated, probably due to damage or loss of CA1 pyramidal cells. On the other hand, the TASK2 expression was significantly upregulated in the dentate granuler and CA3 pyramidal cell layers and endfeet of perivascular astrocytes[28]. These findings suggest that upregulation of TASK2 channels may make a contribution to adaptive responses by inducing hyperpolarization and reducing seizure activity.

Ion channels are essential for the regulation of excitability in the central nervous system[3]. It is believed that various inherited diseases associated with abnormal excitability of the affected neurons are caused as a result of mutations in ion channel encoding genes[2]. Several studies reported the discovery of epilepsy-related mutations in genes encoding TASK channel proteins. Childhood absence epilepsy is an idiopathic, generalized, nonconvulsive epilepsy that occurs in otherwise normal children. The *KCNK9* gene coding for the TASK3 channel is present on chromosome 8 in a locus that shows positive genetic linkage to the human absence epilepsy phenotype[29]. Furthermore, in the genetic absence epilepsy rats from Strasbourg (GAERS), an additional alanine residue in a polyalanine tract within the C-terminal intracellular domain was detected in the *KCNK* gene. For this reason, *TASK3* channels were regarded as a promising candidate gene for absence epilepsy. However, there were no significant differences in the physiological properties between the wild-type and mutant *TASK3* channels[30]. In addition, leak K^+ currents were almost similar between thalamocortical neuros in GAERS and nonepileptic animals[30]. These observations suggest that *TASK3* gene was not associated with absence epilepsy. On the other hand, a mutation analysis of the *TASK3* gene was performed in patients with children and juvenile absence epilepsy[31]. Only one silent polymorphism was detected in exon 2 of the TASK-3 coding region. However, since there was no relationship between the exon 2 polymorphism and absence epilepsy[31], the human TASK-3 appears not to be involved in the absence epilepsy.

Apoptosis

During brain development, the cell excitability is an important determinant for neuronal survival and proliferation[32]. K^+ channels are responsible for the resting membrane potential and action potential duration. Activation of K^+ channels results in membrane hyperpolarization, which significantly influences neuronal death or survival. It has been demonstated that activation of K^+ channels induced neuronal apoptotic cell death[33,34] whereas it protected neurons from ischemia[35] and glutamate-induced cell death[36]. Previous studies revealed that the expression of TASK channels may substantially affect cell viability in either direction[37]. It has been demonstrated that TASK3 channels are responsible for K^+-dependent apoptosis in cultured cerebellar granule neurons. Neuronal death was caused by apoptosis when cerebellar granule neurons were cultured *in vitro* in physiological K^+ concentration, but was prevented when they were cultured in high K^+ concentration. The cell death of granule neurons was also suppressed by pharmacological inhibition

of TASK3 channels with extracellular acidosis and ruthenium red. The cell death was in parallel with the expression level of TASK3 channels[37]. These results indicate a direct relationship between the activity of TASK3 channels and programmed cell death that is necessary for shaping the appropriate cerebellar structure. The authors have also shown that genetic transfection of TASK channel subunits into cultured hippocampal neurons induced apoptotic effect. On the other hand, viral overexpression of TASK3 in cultured hippocampal slices increased cell survival during cellular stress conditions such as an oxygen-glucose deprivation injury[38]. These results suggested that the activation of TASK3 channels can also be protective in neurons under cellular stress conditions.

Learning, memory and cognition

TASK1 and TASK3 channels are widely expressed in the central nervous system. Therefore, it is suggested that the deletion of TASK1 and/or TASK3 channels affects learning and memory. However, in TASK1 KO mice, the higher brain functions were almost similar to the wild-type mice[39]. For example, there were no appreciable differences in anxiety-related behavior and stress-induced hyperthermia between the wild-type and TASK1 KO mice, although the deletion of TASK1 enhanced the sensitivity to thermal nociceptive stimuli[39]. By contrast, TASK3 KO mice exhibited pronounced behavioral changes in relation to memory functions compared with the TASK1 KO mice[40]. In T-maze spontaneous alternation test, the performance in the TASK3 KO mice was poorer compared to the wild-type mice, indicating that working memory was impaired. In addition, during training for the Morris water-maze spatial memory task, the TASK3 KO mice were slower to find the hidden platform, suggesting the impairment of learning[40]. In TASK3 KO mice, the action potential generation and sustained repetitive firing to suprathreshold depolarization were impaired in the granule neurons[41]. Since long term synaptic changes induced by spike activity are believed to underlie learning and memory[42], it is possible that the reduced working memory is ascribed to the impaired spike activity caused by the deletion of TASK3.

CONCLUSION

TASK channels produce background K^+ currents that are time- and voltage- independent, and play crucial roles in setting the resting membrane potential and controlling the K^+ homeostasis. These channels are distributed abundantly in the central nervous system and involved in neurological disorders. Therefore, TASK channel subunits can serve as the molecular targets for treatment of neurological disorders. However, the roles of TASK channels in neurological disorders are just beginning to be investigated. Future studies on the TASK channels will be able to provide even more revealing insights into the neurological disorders.

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Thrombolysis for mild stroke

Hai-Chao Ji, Le-Tian Yang, Fu-Ling Yan

Hai-Chao Ji, Le-Tian Yang, Fu-Ling Yan, Department of Neurology, Zhongda Hospital affiliated to Southeast University, Nanjing 210009, Jiangsu Province, China

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Correspondence to: Fu-Ling Yan, Professor, Department of Neurology, Zhongda Hospital affiliated to Southeast University, 87 Dingjia Qiao, Nanjing Hunan Road, Nanjing 210009, Jiangsu Province, China. yanfuling218@163.com
Telephone: +86-25-83262251
Fax: +86-25-83272011

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Abstract

The term "mild stroke", or "minor stroke" refers to the acute ischemic stroke patients with mild and nondisabling symptoms. Currently there is still no unanimous consensus on the exact definition of mild stroke. Patients with mild stroke are assumed to have a good prognosis in natural course, so they are routinely not given thrombolysis despite early emergency department arrival. Recent studies have revealed that, however, approximately one third of so-called mild stroke patients who are not treated with thrombolysis have significant disability whereas those treated are more likely to achieve a good recovery. Thus excluding all mild strokes from thrombolysis is probably not justified. Those mild stroke patients who are likely to experience early deterioration or end with disability are mostly characterized by imaging findings. Therefore, selected patients with these characteristics based on neuroimaging to be given thrombolysis might be more justified. Meanwhile, new definition should be developed to exclude those who are at a higher risk of poor outcome. Applying information from imaging may make it come true. Using neuroimaging information to define mild stroke and select patients with mild symptoms to thrombolysis may be a future direction.

Key words: Definition; Mild stroke; Minor stroke; Neuroimaging; Thrombolysis

Core tip: Clinically, mild stroke patients are routinely excluded from thrombolysis, for the considering that they are too mild and expected to have a good outcome even left untreated. Recent studies showed that mild strokes might also benefit from thrombolysis. However, unlike major stroke, about two thirds of mild stroke patients will have good outcome in nature course; about the one third will end with poor outcomes but they are found to be mostly characterized by imaging features. So we proposed that neuroimaging-based approaches to define mid stroke and selecting mild stroke patients to thrombolysis may be future directions.

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INTRODUCTION

Evidences have confirmed that thrombolysis is the

most effective therapeutic approach for acute cerebral infarction[1,2]. However, a number of stroke victims arriving in hospital within the time window are withheld thrombolytic treatment for several contraindications. "Mild stroke", or "minor stroke", is one of the most common reasons[3]. Yet there is no unanimous consensus on the exact definition for mild stroke and whether mild stroke patients should be given thrombolysis remains a widely controversial issue as the pivotal studies on reperfusion treatment routinely excluded such patients, leading to scarce data available from randomized controlled trials concerning the optimal management. Some studies in the recent reported that patients with mild stroke might also benefit from thrombolysis. This may bring remarkable public health impact as approximately half of the patients with an acute stroke are categorized as mild stroke[4]. We therefore write this review to elaborate the current researches on mild stroke including the definition, the present situation in the treatment and especially the clinical researches on thrombolysis for mild strokes. We also discuss potential approaches to define or treat mild strokes and ultimately hope that a better understanding of mild stroke will foster the development of innovative therapeutic strategies for mild stroke patients.

WHAT IS MILD STROKE?

A deficit measured on the National Institutes of Health Stroke Scale score of ⩽ 3 is the most commonly used definition

The term "minor stroke" was first mentioned 40 years ago by Perdue *et al*[5] referring to the mild neurologic deficits preceding catastrophic cerebral infarction. Since then, the definitions used to identify minor stroke or mild stroke varied from one study to another and some even did not provide a definition[6]. Majority of the existing definitions are based on the symptoms or baseline National Institutes of Health Stroke Scale (NIHSS) in acute stage (Table 1). Regardless of the exact definitions, most these given definitions share an identical meaning - acute ischemic stroke patients with mild and nondisabling symptoms. A deficit measured on the NIHSS score of ⩽ 3 seems to be the most commonly used definition by literature available[6].

An ideal definition of a "minor stroke", just as Fischer *et al*[6] assert, should reflect the following 5 aspects: "(1) it should capture patients with mild and nondisabling symptoms in acute stage and favorable short-term and medium-term outcomes; (2) it should be valid for different subgroups of stroke patients; (3) it should imply both qualitative and quantitative dimensions; (4) it should be simple and useful in daily clinical practice; and (5) it should not overlap with the definition of a transient ischemic attack". Fischer *et al*[6] also assessed the 6 commonly used definitions and concluded that "NIHSS ⩽ 3" is one of the two most suitable (the other is "score ⩽ 1 on each NIHSS item and normal consciousness"). However, it also has several important limitations: (1) NIHSS score of 3 could represents a severe deficit in one NIHSS item or mild deficits in several items. Some physicians generally consider the prior situation more severe than the latter; (2) using 3 as a specific cut point might be arbitrary because the difference of 90 d outcome between patients with a NIHSS score of 3 and 4 is not evident[7]; (3) what's more, approximately one third of patients with an NIHSS score of ⩽ 3 can not be discharged home and a quarter have a poor outcome[8]. As a result, the definition of NIHSS ⩽ 3 could not well reflect the real severity and outcome of acute ischemic patients with mild symptoms.

Patients with mild stroke symptoms as defined by an NIHSS ⩽ 5 are most commonly excluded from thrombolysis in clinical practice and trials

The definition of stroke severity is clinically relevant as "mild stroke" which is a frequently mentioned contraindication. However, it is never clarified and left to the clinicians' judgement although "acute ischemic stroke with a baseline NIHSS ⩽ 3" is a widely applied definition. Thus when stratifying patients based on the severity of the neurologic deficit, clinicians find themselves in hesitation: is an NIHSS of 3 mild but an NIHSS of 4 or 5 not? National Institute of Neurological Disorders and Stroke rt-PA Stroke Study[1] and III European Cooperative Acute Stroke Study[2] exclude NIHSS scores of < 5 from enrollment on the presumption that this represents the mild subgroup. According to the hospital records from 16 adult area hospitals in a study by Khatri *et al*[16], among 437 patients with acute ischemic stroke that presented to emergency departments within 3.5 h, 247 (57%) had a base line NIHSS ⩽ 5 and only 4 were treated with rt-PA. Moreover, in the American Heart Association Get With the Guidelines Registry (GWGR) nationwide program from 2003 to 2009, among 73044 cases arriving to emergency departments within 2 h of symptom onset but excluded from rt-PA treatment, 29612 (41%) were not treated solely due to mild or improving symptoms, and 75% of these had a baseline NIHSS < 5[3]. Many of the stroke patients who are regarded too mild to treat actually have a higher baseline NIHSS than 3. Mild strokes with a NIHSS ⩽ 5 at baseline are more likely to be excluded from thrombolysis in clinical practice and trials[17]. In this aspect, the definition of NIHSS ⩽ 5 can better reflect the clinical profile that a large number of patients are excluded from thrombolysis just because of the mild symptoms. Hence several eminent epidemiological studies employed this definition[4,11,12].

OUTCOME OF PATIENTS WITH MILD STROKES MIGHT NOT BE AS BENIGN AS GENERALLY ASSUMED

MIS are excluded from thrombolysis because they are expected to have good functional outcome. However,

Table 1 Most commonly used definitions for mild stroke by literature

Based on baseline NIHSS	All patients with baseline NIHSS ≤ 3[6-9]
	All patients with baseline NIHSS ≤ 4[7,10]
	All patients with baseline NIHSS ≤ 5[4,11,12]
	All patients with baseline NIHSS ≤ 6[7,13]
	All patients with baseline NIHSS ≤ 9[14]
	All patients with a score 0 or 1 on every baseline NIHSS score item and normal consciousness[6,14]
Based on syndromes	All patients with a lacunar-like syndrome (presumed small vessel occlusive disease) such as pure sensory syndrome, pure motor hemiparesis, sensorimotor syndrome, ataxic hemiparesis, and dysarthria-clumsy hand syndrome[6,14]
	All patients with only motor deficits (can include dysarthria or ataxia) with or without sensory deficits. These patients can have only a combination of motor, coordination, and sensory deficits without any deficits in the spheres of language, level of consciousness, extinction or neglect, horizontal eye movements, or visual fields, deficits generally ascribed to larger territories of focal ischemia[6,14]
	All patients with baseline NIHSS in the lowest (least severe) quartile of severity (NIHSS ≤ 9), excluding all patients with aphasia, extinction, or neglect, or any points on the level-of-consciousness questions[6,14]
Based on imaging	Major stroke: A proximal cerebral artery occlusion on the CTA or MRA; if no occlusion, imaging evidence of significant parenchymal ischemia on NCCT or DWI[15]
	Minor stroke: all of the others except major stroke[15]

NIHSS: National Institutes of Health Stroke Scale; CTA: Computered tomography angiography; MRA: Magnetic resonance angiography; NCCT: Non-contrasted computered tomography; DWI: Diffusion-weighted imaging.

studies indicate that outcome of patients with mild strokes might not be as benign as generally assumed. Khatri *et al*[17] reviewed a prospective cohort of 136 consecutive patients with mild deficits (NIHSS ≤ 5), 40 (29%; 95%CI: 22%-38%) had poor outcomes (modified Rankin Scale score 2-6) at 90 d and early worsening within 5 d were more common among those with poor outcome; Smith *et al*[3] analyzed the outcome of 29200 patients of mild stroke enrolled in GWGR programme who were excluded from thrombolysis, 28.3% were unable to be discharged home and 28.5% were unable to ambulate without assistance at discharge; Multiple studies have confirmed that approximately 25% to 35% of these patients are disabled at the time of discharge or 90 d despite presenting with mild neurological deficits at acute phase.

TREATING MILD STROKES WITH RT-PA - GRADUALLY ACCEPTED BY CLINICIANS

Mild stroke is one of the most common exclusions for thrombolysis. Prior studies have estimated that 30% to 50% of acute ischemic stroke patients arriving within 3 h of symptom onset are not treated with rt-PA just because of "mild stroke" or "rapidly improving stroke symptoms"[3,18]. However, there is a lack of criteria about what is "mild stroke" and such a contraindication is consensus-based, not evidence-based. Across the Specialized Program of Translational Research in Acute Stroke (SPOTRIAS) network, there was a significant variability in the proportion of patients with mild stroke (NIHSS score 0-3) treated with rt-PA, which ranged from 2.7% to 18.0% across the entire consortium[19]. The main reason is the threshold for the decision to treat a mild stroke differs between physicians at the various centers and between centers overall. In the recently published Promoting Acute Thrombolysis in Ischemic Stroke trail, mild stroke was a less frequent ambiguous contraindication in the intervention hospitals compared with the nonintervention ones (17% *vs* 26%)[20]. All these phenomena reflect a paucity of data on how to best treat mild stroke patients and highlight the demand for a randomized trial to clarify the effect of thrombolysis for mild stroke patients.

On the other hand, having recognized the poor outcomes of mild stroke, many medical centers realize that it is unreasonable to leave them untreated and sometimes they inform patients of the medical knowledge about thrombolysis and may follow their choice[21]. Thus an increasing number of patients with mild stroke are treated. The proportion of mild strokes (NIHSS score 0-3) treated with rt-PA increased from 4.8% in 2005 to 10.7% in 2009 across the SPOTRIAS network[19]; In Sweden, an increase in the proportion of patients with mild stroke (NIHSS ≤ 5) treated with thrombolysis has contributed to rising overall thrombolysis rates - the proportion with mild stroke among patients treated with thrombolysis increased from 22.1% in 2007 to 28.7% in 2010[11]; Over the past two decades period, the initial severity of stroke patients enrolled in the large clinical trails has declined gradually[1,2,22]. These trends may reflect the trend toward the use of thrombolytic agents in patients who have less severe acute ischemic stroke. Many investigators therefore proposed that current thrombolysis guidelines need revision[21].

DOES MILD STROKE BENIFIT FROM THROMBOLYSIS?

Currently no randomized, placebo-controlled trial has yet been implemented to test the efficacy and safety of rt-PA administered in patients with mild stroke. Some studies that retrospectively analyse the outcome of mild stroke patients received thrombolysis have attempted to evaluate the safety and efficacy. Some show that the patients treated with thrombolysis tend to aquire better outcomes, while the others do not show advantages. However, nearly all the studies show that the rate of

Table 2 Studies on thrombolysis in mild stroke

Ref.	Definition of mild stroke	Patients		Favorable outcome (t-PA *vs* Placebo)	*P*	SICH (t-PA *vs* Placebo)	*P*
		rt-PA	Placebo				
NINDS rt-PA Stroke Study Group[14]	Score ≤ 1 on each NIHSS item and normal consciousness	21	7	100% *vs* 86%	< 0.02	0% *vs* 0%	-
	Presumed small-vessel stroke	51	30	69% *vs* 60%	< 0.02	4% *vs* 0%	-
	Only motor deficits ± sensory deficits	220	219	61% *vs* 46%	< 0.02	3% *vs* 0.5%	-
	NIHSS score ≤ 9, minus all with aphasia, extinction/neglect, or any points on the level of consciousness questions	97	76	81% *vs* 74%	< 0.02	3% *vs* 0%	-
	NIHSS ≤ 9	99	78	82% *vs* 74%	< 0.02	3% *vs* 0%	-
Köhrmann *et al*[10]	NIHSS ≤ 4	32	-	94%	-	0%	-
Hassan *et al*[13]	NIHSS ≤ 6	27	24	92.6% *vs* 50%	< 0.03	3.7% *vs* 4.2%	1
Hassan *et al*[23]	NIHSS ≤ 10	52	98	74% *vs* 34%	< 0.009	2% *vs* 3%	-
[1]Steffenhagen *et al*[24]	NIHSS ≤ 5	78	16	74.7% *vs* 81.3%	0.75	2.6% *vs* -	0.572
[2]Khatri *et al*[25]	NIHSS ≤ 5	42	16	78.6% *vs* 81.3%	-	2.4% *vs* 0%	-
[2]Huisa *et al*[26]	NIHSS ≤ 5	59	74	57.6% *vs* 68.9%	0.871	5%	-
[3]Helsinki Stroke Thrombolysis Registry[7]	NIHSS 0-2	58	-	88%	-	0%	-
	NIHSS 3-4	194	-	86%	-	2.6%	-
	NIHSS 5-6	236	-	78%	-	2.1%	-
Greisenegger *et al*[27]	NIHSS ≤ 5	445	445	41% *vs* 29%	< 0.001	2.5% *vs* 0%	-
Urra *et al*[28]	NIHSS ≤ 5	119	84	83% *vs* 81%	> 0.05	0% *vs* 0%	-
Logallo *et al*[29]	NIHSS ≤ 5	158	1633	38% *vs* 31%	0.07	1.9% *vs* 0.1%	< 0.001

[1]Steffenhagen *et al*[24] compared the functional outcome of mild stroke patients using the modified Rankin scale (mRS) with severe (NIHSS score > 5) groups. Symptomatic intracerebral hemorrhage was low (2.6% *vs* 4.7%; *P* = 0.572). Favorable outcome (mRS score < 2) at 3 mo was more frequent (74.7% *vs* 34.7%; RR = 2.2, 95%CI: 1.8-2.5, *P* < 0.001) and mortality rate was lower (8% *vs* 22.9%; RR = 0.35, 95%CI: 0.16-0.76, *P* = 0.002). Favorable outcomes were not different (81.3% *vs* 74.7%, mRS score < 2, *P* = 0.75) compared to a placebo-treated group with baseline NIHSS scores ≤ 5 (*n* = 16) from the NINDS t-PA trial; [2]In Khatri's study, 4 of the 58 minor strokes had baseline disability (mRS > 2), and all were in the rt-PA group. In Huisa's study, the NIHSS score at admission was higher in the t-PA treated group compared to that in untreated group (3.4 ± 1.4 *vs* 1.9 ± 1.3; *P* < 0001). Thus the selective bias in the two studies might underestimate the effects of thrombolysis; [3]In Helsinki Stroke Thrombolysis Registry, they did not give the definition of mid stroke. Fifty-eight mild stroke patients with NIHSS score 0-2 were treated with thrombolysis only when hyperdense cerebral artery sign, artery occlusion on computed tomography (CT) angiography, or perfusion deficit on perfusion CT scan presented. NIHSS: National Institutes of Health Stroke Scale; t-PA: Tissue plasminogen activator; SICH: Symptomatic intracranial hemorrhage.

intracranial hemorrhage will not increase significantly in rt-PA groups, which indicates that thrombolysis for mild stroke should be safe (Table 2).

HOW TO TREAT MILD STROKE? - NEUROIMAGING-GUIDED THROMBOLYSIS FOR MILD STROKE MAY BE A POTENTIAL WAY

Unlike major stroke, about two thirds of mild strokes have good functional outcomes in their natural course. Considering the probability that these patients may not benefit much but will be exposed at risks of hemorrhage if given rt-PA, it seems to be not reasonable if all mild strokes are given thrombolytic therapy. On contrary, those who will have a poor outcome and can be figured out ideally might derive the greatest benefit from thrombolysis if treated. A number of studies have tried to find out the the causes and characteristics of mild stroke patients with poor outcomes.

Ohara *et al*[30] retrospectively studied the clinical data of mild strokes (NIHSS ≤ 5) who presented within 3 h after onset and did not receive intravenous rt-PA and found that major vessel occlusion (OR = 6.90; 95%CI: 1.31-47.51; *P* = 0.022) and NIHSS > 3 (OR = 8.00; 95%CI: 1.20-79.31; *P* = 0.031) were independent predictors of poor outcomes. Rajajee *et al*[31] revealed that persisting large-vessel occlusion substantially increases the risk of early worsening (OR = 18; 95%CI: 1.6-209; *P* = 0.02) and poor functional outcome(OR = 7; 95%CI: 1.2-38; *P* = 0.04) in mild stroke patients(NIHSS ≤ 5) while Coutts *et al*[32]'s study indicated that intracranial or extracranial vessel occlusion or ≥ 50% stenosis was associated with poorer outcome (RR= 2.92; 95%CI: 1.81-4.71). A study in Changhua, Taiwan, also demonstrated that mild or rapidly improving patients with initial NIHSS score ≥ 3 had high risk of unfavorable outcome (OR = 5.95; 95%CI: 1.10-32.12)[33].

Moreover, several studies have obtained amazing results through analyzing multimodal MRIs of mild strokes. A study by Khatri *et al*[17] showed that mild strokes (NIHSS ≤ 5) with poor outcome had larger DWI infarcts at baseline and more frequent lesions growth and NIHSS worsening from baseline to 5 d, increase in DWI infarct volume (OR = 3.57; 95%CI: 1.17-10.9; *P* < 0.03) was an independent predictor of poor 90-d outcome. Similarly, in Asdaghi *et al*[34]'s study, early "recurrence" of mild stroke (NIHSS ≤ 5) was much more likely in patients with larger baseline diffusion-weighted imaging (DWI) or perfusion-weighted imaging (PWI) lesions and all new lesions developed within the

baseline PWI infarcts. Interestingly, both Asdaghi *et al*[34] and Rajajee *et al*[31]'s studies showed that baseline large DWI lesion or DWI-PWI mismatch were frequently accompanied by large vessel occlusions in those mild patients who underwent early neurologic deterioration with infarct expansion and ended with poor functional status. These findings suggest that majority of "recurrent" events or deteriorations over the first few days after the mild strokes are related to progression of the original infarct within the territory of the penumbral deficit due to consistent vessel occlusion, rather than new cerebrovascular events[35]. Thrombolysis may restrain this progression and bring potential benefit.

Studies above illustrate that baseline NIHSS > 3, major vessel occlusion or severe stenosis, relatively large DWI or PWI lesions are strongly associated with early deterioration and poor prognosis, which suggests that selecting mild strokes with these characteristics to treat might be more justified. Specifically, strokes with baseline NIHSS > 3 but without contraindications perhaps all should be given thrombolysis[36], while patients with baseline NIHSS $\leqslant$ 3 should be given thrombolysis or other reperfusion therapies as well on condition that major vessel occlusion, relatively large DWI and PWI lesions or significant DWI/PWI mismatch be visualized with rapid neuroimaging methods[37]. Certainly, justification of this selective approach awaits clinical validation in more studies.

RETHINK THE DEFINITION OF MILD STROKE

To supplement NIHSS, many studies have proposed imaging-based methods to evaluate and classify acute cerebral infarction[38]. However, few such studies have yet to be performed for mild stroke. Torres-Mozqueda *et al*[15] proposed a neuroimaging-based ischemic stroke classification system - Boston Acute Stroke Imaging Scale (BASIS). The rationale underlying this classification system is that if proximal cerebral artery occlusions are identified on the computered tomography angiography or Magnetic Resonance Angiography, or significant parenchymal abnormalities are identified by examination of the non-contrasted CT or diffusion MR imaging, patients will be classified as having a major stroke. All of the other patients are classified as having a mild stroke. Compared to NIHSS and other widely accepted imaging-based scale such as Alberta Stroke Programme Early CT Score[39], BASIS is found to be highly effective in predicting outcomes and applicable to both anterior and posterior circulation strokes[40]. What's more, it focuses on the primary cause of the infarct - arterial occlusion[15], which will give physicians more clues than NIHSS to determine whether thrombolysis should be given or not, especially for patients with mild symptoms. Clearly, definition solely based on baseline NIHSS or symptoms seems not to be a good choice. Using neuroimaging methods might be more reasonable and superior to using NIHSS in terms of suggesting prognosis and helping to guide therapy in individual patient. Advances in the field of neuroimaging have made it quite feasible and achievable[38], but few such studies have yet to be performed and it may be a future direction to define mild stroke.

CONCLUSION

The definition of mild stroke is not uniform yet. The term "mild stroke" or "minor stroke" might not be suitable for all those stroke patients who are currently deemed too mild to treat as a significant percentage of them will be disabled if left untreated. Definitions on the basis of NIHHS solely might not be a good choice. An ideal definition should better differentiate those who are at a higher risk for clinical deterioration and poor prognosis from the patients presenting with mild neurological deficits or low NIHSS. Applying information from neuroimaging may help it come true.

The available studies on rt-PA for mild stroke inform us that thrombolysis may be beneficial and with minimal side effects. Considering the fact that most of them have favorable functional outcomes in their natural course, selecting patients who are most likely to be early worsening and disabled to treat might be more justified. Current studies have identified some characteristics of these patients and efforts of neuroimaging-guided thrombolysis in mild stroke have been made. We think that it will be a future direction for treating mild strokes.

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EDITORIAL

Hypocretin (orexin) pathology in Alzheimer's disease

Thomas C Thannickal

Thomas C Thannickal, Department of Psychiatry and Biobehavioral Sciences, University of California, Los Angeles, CA 90025, United States

Thomas C Thannickal, Veterans Administration Greater Los Angeles Healthcare System, Neurobiology Research (151A3), North Hills, CA 91343, United States

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Correspondence to: Thomas C Thannickal, PhD, Associate Researcher, Veterans Administration Greater Los Angeles Healthcare System, Neurobiology Research (151A3), North Hills, 16111 Plummer St., CA 91343,
United States. thomastc@ucla.edu
Telephone: +1-818-8917711
Fax: +1-818-8959575

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Abstract

Alzheimer's disease (AD) is a growing health problem. It has enormous public health impact. Sleep problems show an early component of this disease. Hypocretin has a major function in sleep-wake cycle. The total number of hypocretin neurons in the normal humans ranges from 51000-83000, located exclusively in the hypothalamus. Deficiency in hypocretins neurotransmission results in narcolepsy, Parkinson's disease, and other neurological and psychological disorders. Cerebrospinal fluid (CSF) hypocretin levels were directly related with t-tau protein amount in AD. Increased hypocretin CSF in AD suggest that hypocretin is involved in the mechanism of AD pathology.

Key words: Hypocretin; Orexin; Alzheimer's disease; Neurological disorders

Core tip: Hypocretin plays an important role in the control of sleep-wake cycle. Increased hypocretin levels in Alzheimer's disease patients suggest hypocretin system is involved during development of the disease symptoms.

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INTRODUCTION

The hypocretins were discovered in 1998 by two groups[1,2]. One group named hypocretins because of hypothalamic origin and similarity with the secretin[1]. The other group named Orexins because these neurotransmitters stimulated food intake[2]. Their projection target suggests hypocretins have a neuromodulatory role in neuroendocrine and homeostatic functions[3,4]. The distribution of hypocretins neurons in human hypothalamus is shown in Figure 1. Hypocretin fibers and receptors are found throughout the brain[3,5,6]. Hypocretins

Table 1 Important findings shows role of hypocretin in Alzheimer's disease

Ref.	Year	Major findings
Friedman *et al*[28]	2007	Increased wake fragmentation found in those with lower hypocretin-1
Kang *et al*[29]	2009	Amyloid-beta dynamics are regulated by hypocretin and the sleep-wake cycle
Fronczek *et al*[27]	2011	40% hypocretin cell loss in Alzheimer's disease
Slats *et al*[30]	2012	Association between hypocretin-1 and amyloid-β42 cerebrospinal fluid levels
Roh *et al*[8]	2014	Modulation of hypocretin and its effects on sleep to modulate Aβ pathology
Liguori *et al*[7]	2014	Increased hypocretin level correlates with sleep disruption and cognitive decline in Alzheimer's disease

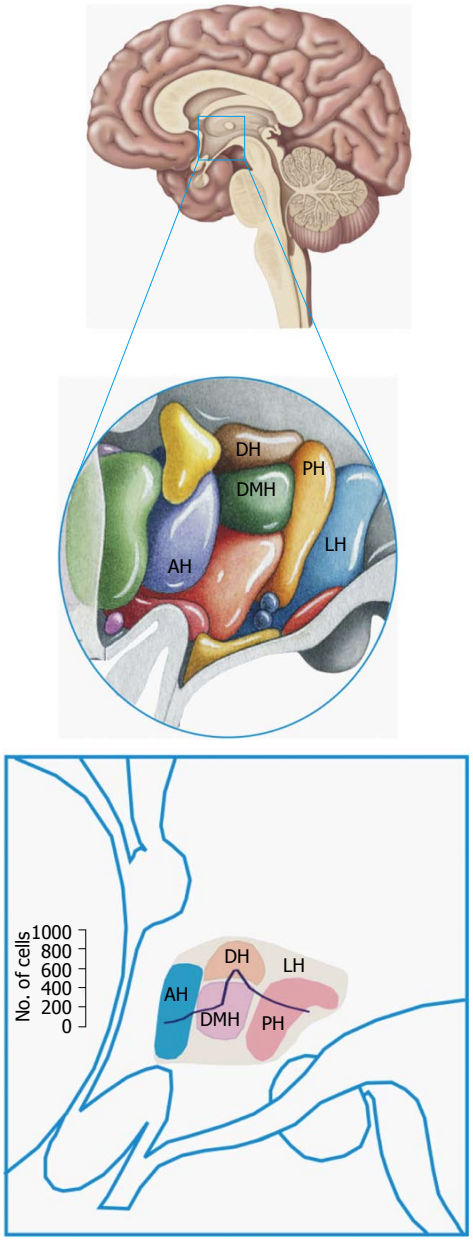

Figure 1 Distribution of hypocretin neurons in human hypothalamus. The normal distribution of hypocretins cells in the hypothalamus is limited to AH, DH, DMH, LH and PH. AH: Anterior hypothalamus; DH: Dorsal hypothalamus; DMH: Dorsomedial hypothalamus; LH: Lateral hypothalamus; PH: Posterior hypothalamus.

loss in narcoleptics opened importance of hypocretin system in health and disease[5]. New findings show the role of hypocretin in the pathogenesis of Alzheimer disease (AD)[7,8].

HYPOCRETIN AND NEUROLOGICAL DISORDERS

Narcoleptic patients have low or undetectable cerebrospinal fluid (CSF) hypocretin[9]. The pathological studies revealed 85%-95% loss of hypocretins cells in narcoleptics with cataplexy[10]. Maximum cell loss was occurred in the posterior and tuberomammillary nucleus[11,12]. Decreased CSF hypocretin were reported in, idiopathic hypersomnia, hypothalamic neoplasms and acute disseminated encephalomyelitis[13-17]. Higher CSF hypocretin were found in restless legs syndrome[18]. Lower hypocretin CSF were reported in patients with multiple sclerosis[16], Niemann Pick disease type C[19] and Whipple's disease[20]. Hypocretin cell loss was found in Parkinson disease patients[21,22]. Benarroch *et al*[23] reported 70% loss of hypocretins cells in multiple system atrophy patients. In Huntington's disease 30% loss of hypocretins cells occurred[24]. Bauman *et al*[25] found hypocretins cell loss in TBI patients with severe injury. There was reduced fluctuations of hypocretins CSF in depression patients[26].

DYSREGULATION OF HYPOCRETIN SYSTEM IN AD

Number of hypocretins cells in AD patients were reduced by 40%[27]. AD patients with lower hypocretins-1 showed increased wake fragmentation[28]. Kang *et al*[29] reported the role of hypocretins and sleep in amyloid beta dynamics. The link between mean amyloid beta 42 and hypocretins suggests a relationship between AD pathology and hypocretin disturbance. The important findings related to the role of hypocretins in AD are summarised in Table 1. With symptom progress AD patients had increased hypocretins levels. The hypocretin levels in AD were associated with tau protein and sleep impairment. Hypocretin output and function seem to be over expressed with disease[8]. A few literature reports showing that[30,31] there was no decrease in CSF hypocretin levels. These studies considered smaller samples including some cases, patients receiving psychiatric medications, which may influence hypocretin neuronal activity and output. Liguori *et al*[7], results are in contrast to Fronczek *et al*[27], study reporting decreased

hypocrein neurons and CSF levels in AD patients. This difference may be related to the fact that Ligouri *et al*[7], performed *in vivo* study, whereas Fronczek *et al*[27], were used pathological tissues from advanced AD patients. Roh *et al*[8], found that hypocretin knockout animals slept for longer time and lower amyloid-beta. These studies show the importance of hypocretin system in AD.

AD AND NARCOLEPY

The core sleep problems in AD and narcoleptic patients are partly resemble. Hypocretin may have an important function in the pathological mechanism of AD. In narcoleptic patients with cataplexy have 90% of hypocretins cell loss and undetectable level of CSF hypocretin. If hypocretins mediates AD symptom progress, narcolepsy patients should be protected against AD pathology. The neuropatholgical records of twelve narcolepsy with cataplexy showed that thirty three percentages of these narcoleptics had AD pathology which is comparable to the prevalence in general population[32]. This report shows that severe loss of hypocretin neurotransmitter does not protect from AD.

HYPOCRETIN AS A CSF BIOMARKER

Higher CSF t-tau protein levels mark the AD neurodegeneration. Increased t-tau levels represent a sign of rapid cognitive decline because they have been faster more pronounced neuronal degeneration, supporting the transition from early to more advanced disease stages[33]. CSF hypocretin levels were directly correlated with t-tau protein levels in AD[8,34] . This finding suggests that higher hypocretin levels may be related to rapid tau-mediated degeneration in AD. The pathogenesis of AD may therefore involve dysregulation of the hypocretins system, with over expression of hypocretins output and function.

CONCLUSION

With a rising prevalence of AD around the world, there is an urgent need to identify opportunities for prevention and treatment of the disease. Hypocretin may have a role in the pathological process leading to AD. The pathogenesis of AD may therefore involve dysregulation of the hypocretins system, with over expression of hypocretins output and function, manifested as sleep disturbance and associated with progressive neurodegeneration. Further studies on the importance of hypocretin during the process of AD could lead to new preventive and therapeutic findings.

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EDITORIAL

Human T-lymphotropic virus type 1-associated myelopathy/tropical spastic paraparesis: Clinical presentation and pathophysiology

Jean-Pierre Louboutin

Jean-Pierre Louboutin, Department of Basic Medical Sciences, University of the West Indies, Mona Campus, Kingston 7, Jamaica

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Correspondence to: Jean-Pierre Louboutin, MD, PhD, Department of Basic Medical Sciences, University of the West Indies, Mona Campus, Kingston 7, Jamaica. jplouboutin@hotmail.com
Telephone: +1-876-3680554

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Abstract

Human T-cell lymphotropic virus type 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a slowly progressive neurodegenerative disorder in which lesions of the central nervous system cause progressive weakness, stiffness, and a lower limb spastic paraparesis. In some cases, polymyositis, inclusion body myositis, or amyotrophic lateral sclerosis-like syndromes are associated with HTLV-1. TSP was first described in Jamaica in 1888 and known as Jamaican peripheral neuritis before TSP was related to HTLV-1 virus, the first retrovirus being identified, and the disease is since named HAM/TSP. There is no established treatment program for HAM/TSP. Prevention is difficult in low-income patients (*i.e.*, HTLV-1 infected breast feeding mothers in rural areas, sex workers). Thus, there is a need for new therapeutic avenues. Therapeutic approaches must be based on a better understanding, not only of clinical and clinicopathological data, but also of the pathophysiology of the affection. Consequently, a better understanding of existing or newly developed animal models of HAM/TSP is a prerequisite step in the development of new treatments.

Key words: Tropical spastic paraparesis; Human T-cell lymphotropic virus type-1; Polymyositis; Animal models; Retroviruses; Myelopathy; Human T-cell lymphotropic virus type 1-associated myelopathy; Pathogenesis

Core tip: Human T-cell lymphotropic virus type 1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a slowly progressive neurodegenerative disorder in which lesions of the central nervous system cause progressive weakness, stiffness, and a lower limb spastic paraparesis. There is no established treatment program for HAM/TSP. Prevention is difficult in low-income patients. Thus, there is a need for new therapeutic avenues that must be based on a better understanding, not only of clinical and clinicopathological data, but also of the pathophysiology of the affection. Consequently, a better understanding of existing or newly developed animal models of HAM/TSP is a prerequisite step in the development of new treatments.

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INTRODUCTION

Human T-cell lymphotropic virus type 1 or human T-lymphotropic virus type 1 (HTLV-1), (also called the adult T-cell lymphoma virus type 1) is a retrovirus belonging to the family retroviridae and to the genus deltaretrovirus. HTLV-1 was the first identified human retrovirus and is the etiological agent of two distinct diseases: Adult T-cell Leukemia/Lymphoma (ATL) and HTLV-1-Associated myelopathy/tropical spastic paraparesis (HAM/TSP)[1]. It is estimated that HTLV-1 infects approximately 10-20 millions individuals worldwide[2]. Endemic areas include Japan, Brazil, the Caribbean, inter-tropical Africa, Eastern Europe, and the Middle East. In United States, Canada and Western Europe, it appears that HTLV-1 is mainly found in immigrants. It seems however that there is a cluster of HAM/TSP cases in Romania. One to 5 percent of the individuals infected with HTLV-1 eventually develop ATL, an aggressive malignancy of mature activated $CD4^+$ T cells, characterized by frequent visceral involvement, and opportunistic infections secondary to T cell immunosuppression. One to four per cents of patients infected by HTLV-1 will develop HAM/TSP[2]. TSP was first described in Jamaica in 1888 and was known as Jamaican peripheral neuritis before TSP was related to HTLV-1 in patients from Martinique presenting similar symptoms[1]; the affection was then called HAM. Large cohorts of patients with TSP have been reported in Jamaica[3]. HAM/TSP seems more common in lower socio-economic groups and more prevalent in rural regions. Currently, there is no established treatment program for this disorder. Prevention is difficult in low-income patients (*i.e.,* HTLV-1 infected breast feeding mothers in rural areas, sex workers). Even if the incidence is currently lower due to detection of HTLV-1-positive blood donors, there are still numerous cases of HAM/TSP.

CLINICAL PRESENTATION

Infection of HTLV-1 can be spread through contaminated blood products, passage from mother to child and sexual transmission. Very high rates of transmission of HTLV-1 are observed with blood products containing infected cells, as well as after transplantation of organs from HTLV-1 positive patients. However, blood screening prior to transfusion reduces the rate of transmission through infected blood products. Viral transmission can also occur through the use of injected drugs. Sexual transmission is mostly male to female, and explains the increasing numbers of HTLV-1 positive women, particularly in sex workers. Passage from mother to child can occur within the womb, by perinatal transmission, or *via* breastfeeding, particularly if prolonged after 6 mo of age, and if the proviral load is high in the milk. In Jamaica, HAM/TSP seem to be more frequent in rural areas and in low income patients[3].

HAM/TSP is a slowly progressive neurodegenerative disorder in which lesions in the central nervous system (CNS) predominate in the lower spinal cord and cause progressive weakness, difficulty walking, stiffness, and a lower limb spastic paraparesis. Foot dragging, difficulty running, impairment of ambulation, are present in 60%-80% of cases. Weakness usually begins on one side, then extends to the other side over months or years. Pyramidal signs, paraparesis and urinary symptoms are observed in almost 100% of cases. Legs are uniformly involved but arms are weak in up to 33% of patients. Hyperreflexia of lower limbs and Babinski sign are present in more than 90% of cases. Sensory signs (paresthesia and sensory cord levels) are less frequent (25%-78%). Pain and muscle atrophy are less common. Disability occurs during the first year of the disease. The disease is progressive without remissions. The WHO diagnostic criteria for HAM/TSP require presence of paraparesis (associated or not with sensory and/or autonomic abnormalities), positive serology for HTLV-1, and presence of the virus in the cerebrospinal fluid[4].

In addition to the typical HAM/TSP, there are some neurological manifestations related to HTLV-1, but without the typical form. Manifestations of autonomic dysfunctions (overactive bladder, heart rate and blood pressure dysregulation, erectile dysfunction, constipation), and sensory dysfunction (impaired proprioceptive and vibratory function) can be observed without HAM/TSP. Overactive bladder, characterized by incontinence, nocturnia, and urgency, is one of the most common symptoms in patients without paraparesis. In some cases, overactive bladder precede HAM/TSP by years, being the initial manifestation of the disease. Patients with neurological symptoms not fulfilling the typical HAM/TSP criteria can be classified as possible or probable HAM/TSP[4-6].

Other neurological symptoms linked to HTLV-1, but not related to myelopathy have also been reported. Cognitive dysfunction (impairment of visual and verbal memory, abnormalities of attention, and Mini-Mental State Exam), as well as cerebellar ataxia (mostly vermian cerebellar syndrome, frequently progressing to HAM/TSP, suggesting a spinocerebellar syndrome) have been associated to HTLV-1. Rare amyotrophic lateral sclerosis (ALS)-like syndromes have been described, but they differ from typical ALS by a long-term survival, and the presence of overactive bladder. Polyneuropathy, mostly sensory-motor polyneuropathy, can be associated to HTLV-1. Peripheral neuropathy (predominantly axonal) can be found in 30% of patients with HAM/TSP, but more rarely in patients without HAM/TSP (6%). Cases of cranial mononeuropathy,

usually facial nerve palsy, have been reported; a study in Trinidad and Tobago found that 21% of cases of facial nerve palsy were associated with HTLV-1[4-6]. In some cases, polymyositis, and inclusion body myositis, are related to HTLV-1, but are usually not associated with HAM/TSP. In Jamaica, 85% of patients with polymyositis are HTLV-1-positive[7].

Symptoms usually begin around age 30. Most patients have insidious course progressing over months to years. Median time from onset to use of a cane is 6 years, a walker at 13 years, and a wheelchair at 21 years[8,9]. About 10%-20% progress to severe gait impairment over 1-3 mo. More rapid progression tend to occur in patients older than 50 with a high viral load[8], or after blood transfusion[10], or organ transplantation[11]. However, onset and course are highly variable. Disease usually begins with asymmetric leg weakness and stiffness. Progressively, other leg becomes involved over months or years. Spasticity then becomes more pronounced and impairment of ambulation soon appears[6].

Routine CSF analysis may be normal or show various abnormalities. Glucose level is normal. Protein is elevated in up to 40% of patients. Cell counts are elevated in up to 57% of patients, consisting entirely of mononuclear cells[12]. Elevated intrathecal production of IgG (IgG index, oligoclonal IgG, or CSF IgG synthesis rate) occurs in 20%-85% of cases. Proviral load in CSF cells is higher than peripheral blood mononuclear cells[6]. Brain MRI abnormalities are frequently seen (from 25% to 80% of patients)[13]. T2 protocols are more sensitive to demonstrate lesions appearing as T2 hyperintense signal abnormalities. Lesions, frequently multifocal, are observed in subcortical, periventricular and deep cerebral areas. White matter lesions are also frequent in HTLV-1 carriers, and the lesions in this group are similar to the ones in HAM/TSP patients[13]. electroenceph-alogram (EEG), and pathologic evaluation have shown a widespread involvement of the CNS. Diffuse EEG abnormalities (poor organization or slowing of background activity to that bursts and/or spikes) have been reported in 64% of patients[14]. Evoked potentials, particularly somatosensory evoked potentials (SSEPs) are frequently abnormal[15].

Many disorders can be discussed in patients presenting with progressive or mildly relapsing myelopathy: multiple sclerosis, Lyme disease, vitamin B12 deficiency, human immunodeficiency virus (HIV) infection, spinal cord compression. Konzo is a form of spasticity prevalent in Africa associated with excessive consumption of cassava and chronic cyanide intoxication.

Besides HTLV-1, myelopathies are rarely related to viral infections. Rare cases of tropical spastic paraparesis are caused by HTLV-2. Double infection with HTLV-1/HIV-1 is not infrequent in areas with high prevalence of acquired immune deficiency syndrome[16]. Other viral infections are uncommon causes of acute myelopathies (*e.g.,* poliomyelitis, herpesviruses). Besides poliovirus, flaviviruses (including West Nile virus), enterovirus-71, and cocksackieviruses A and B can induced anterior horn cell necrosis[16].

However, residence in a high seroprevalence endemic area, history of transfusion exposure, or IV drug abuse, or working as a sex worker, is highly indicative of the disease. CSF with inflammation and intrathecal production of IgG, abnormalities of SSEPs, hyperintense T2 signals or spinal cord atrophy on MRI are suggestive as well. Other associated systemic manifestations (*i.e.,* persistent prostatitis, dermatitis, bronchoalveolitis) can lead to the diagnostic that will be confirmed by an immunoassay and Western-blot showing HTLV-1 specific antibodies[4-6,16]. The Western-blot analysis differentiates HTLV-1 from HTLV-2 infection. Study of the CSF is the mandatory step to confirm the diagnosis. Polymerase chain reaction on CSF cells will confirm the diagnosis of CNS infection and help to distinguish HTLV-1 from HTLV-2[4-6,16].

PATHOGENESIS

There are different theories regarding the disease hypothesis. The most widely accepted theory related to HAM/TSP is that of a virally induced, cytotoxic, demyelinating inflammatory process of a chronic and progressive nature affecting the spinal cord. The infection by HTLV-1 triggers an antigen-specific immune response towards the HTLV-1 antigen. Cytotoxic $CD8^+$ T-lymphocytes of the host's immune response release cytokines in an effort to fight the infection. These cytokines facilitate the migration of lymphocytes across the blood-brain barrier (BBB). Demyelination is brought as a result of bystander cell injury, probably by apoptosis of oligodendrocytes. Activated microglia are a prominent feature found in the spinal cord of patients with HAM/TSP[17]. However, the role of microglia is not totally clear. Cells of microglia/macrophage lineage might be one of viral reservoirs in the spinal cords in HAM rat disease[18]. However, this point is somehow still debated. Unlike most other viruses, cell-free HTLV-1 is poorly infectious and efficient infection requires cell-cell contact. In the brain of some HAM/TSP patients, astrocytes are infected with HTLV-1. However, HTLV-1 is primarily found in $CD4^+$ T cells. Although $CD4^+$ T cells are the major reservoir, other hematopoietic cells ($CD8^+$ T cells, B lymphocytes, monocytes, macrophages, dendritic cells) and microglial cells have been infected with HTLV-1[19-23]. There are conflicting reports concerning the potential of HTLV-1 to infect microglial cells[24,25]. In one patient co-infected by HIV-1 and HTLV-1, presenting with HAM/TSP and HAND, HTLV-1 was localized to astrocytes and HIV-1 to microglia/macrophages[26]. In an attempt to study Tax-induced production of cytokines in human microglial cells and astrocytes, transduction of these cells has been done by using lentiviral vectors stably expressing Tax (oncoprotein of HTLV-1) gene. Results show that Tax can up-regulate cellular proinflammatory cytokine expression profile in human microglial cells and human fetal astrocytes[27]. However, HTLV-1 specific $CD8^+$ lymphocytes that secrete the neurotoxic cytokines

interferon-gamma (IFN-gamma) and tumour necrosis factor (TNF) are present[28] and may be responsible for bystander damage[29,30]. Extracellular Tax released from infiltrating T cells could induce cytokine release by microglia and contribute to demyelination and inflammation in the absence of detectable virus[31]. Like in other neurodegenerative diseases, it is possible that, associated with neuroinflammation, oxidative stress plays a role in the pathogenesis of HAM/TSP.

If the pathogenesis of HAM/TSP is still unknown, more data are available concerning the events leading to ATL. The pathogenesis of ATL results from the malignant transformation of CD4-positive cells. Tax induces this transformation by binding to transcription factors to promote transcription of the proviral genome. However, Tax has many other effects from repressing genes involved in DNA repair and activation of apoptosis, to inhibition of proteins involved in tumor suppression[32]. The distribution of proviral integration site is different between asymptomatic carriers, HAM/TSP and ATL patients[33].

ANIMAL MODELS

A number of various animal models have been developed for HAM/TSP. However, none of these models fully recapitulates HTLV-1-associated disease. Injection of immortalized MT-2 cells infected with HTLV-1 has been used in numerous experiments. ICR mice have been immunized with HTLV-I carrier T lymphocytes (MT-2 cell line) and then inoculated intracerebrally with these cells. In this experiment, perivascular cell infiltration was observed diffusely throughout the brain for over 2 wk[34]. HTLV-I antigens were detected in both sides of the cerebral hemisphere and tissue damage consisting of demyelination, axonal degeneration, and astrogliosis was observed most heavily between days 10 and 14[34]. Intraperitoneal (*ip*) inoculation of immortalized MT-2 cells infected with HTLV-1 in newborn WKAH rats can induce a chronic progressive myeloneuropathy with spastic paraparesis linked to apoptosis of oligodendrocytes in anterior funiculi of upper thoracic spinal cord. However, these signs appear 15-22 mo after inoculation[35,36]. The same animal model is characterized by activation of TNF-alpha and pX (area of HTLV-1 genome where genes for Tax and Rex regulatory proteins are located) genes[37,38]. Mononuclear infiltration was seen in the animal model previously described. Activated microglial cells and macrophages were observed 15 mo after HTLV-1 injection in WKAH rats. IFN-gamma can protect against the development of HAM rat disease[39]. If all these results are important, they have been obtained mainly in WKAH rats, mostly injected *ip* few days after birth, and signs appear more than one year after inoculation of immortalized MT-2 cells infected with HTLV-1.

Intravenous (*iv*) injection of whole blood from HAM/TSP patients or cells infected with HTLV-1 gives conflicting results[40-42]. However, these findings support the human evolution of the disease with its expression during adult or older adult age, as observed in rats aged 12 to 15 mo, corresponding on a human scale to 40 to 60 years of age. Besides presenting tumors, Tax (oncoprotein of HTLV-1) transgenic mice can develop a disease characterized by degeneration of oxidative muscle fibers or symmetrical paraparesis of the hind limbs[43]. Inoculation of HTLV-1 in monkeys induced polymyositis[44].

New ways of inoculating HTLV-1 should be investigated. HTLV-1-infected MT-2 cells have been used so far; however, most of the work has been done in newborn WKAH rats injected *ip* with these cells and HAM/TSP appears after at least one year in this model. The BBB might prevent HTLV-1-infected MT-2 to reach the CNS. It has been shown that faster and more reproducible results were obtained in adult mice with a direct intra-cerebral injection of these cells[18], suggesting that BBB might be a critical factor. In fact, it has been shown that human endothelial cells can be infected *in vitro* by HTLV-1[45], and that co-cultures of HTLV-1 and with human brain endothelial cell line leads to loss of tight junction proteins[2]. However, these results have been obtained *in vitro*. One way to circumvent the BBB would be to cause a breach of it for example by using an *ip* administration of mannitol before injecting HTLV-1-infected MT-2[46]. Inoculating cells in the cisterna magna, an area close to the spinal cord, following *ip* injection of mannitol would be less traumatic and more simple than directly into the brain or lateral ventricle[47]. Alternatively, *iv* injection of whole blood of patients with prior administration of mannitol, could be realized[48]. Moreover, it has been shown *in vitro* that BBB is abnormal in HTLV-1 related injury, and the previously described models could mimic these features[2].

In conclusion, more studies are necessary to define the pathophysiology of HAM/TSP[49]. Better animal models can pave the way for novel therapeutic approaches.

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FRONTIER

Mechanical transduction by ion channels: A cautionary tale

Frederick Sachs

Frederick Sachs, UB School of Medicine and Biomedical Sciences, Physiology and Biophysics, Buffalo, NY 14214, United States

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Correspondence to: Frederick Sachs, PhD, UB School of Medicine and Biomedical Sciences, Physiology and Biophysics, 301 Cary Hall, Buffalo, NY 14214,
United States. sachs@buffalo.edu
Telephone: +1-716-8295161
Fax: +1-716-8292569

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Abstract

Mechanical transduction by ion channels occurs in all cells. The physiological functions of these channels have just begun to be elaborated, but if we focus on the upper animal kingdom, these channels serve the common sensory services such as hearing and touch, provide the central nervous system with information on the force and position of muscles and joints, and they provide the autonomic system with information about the filling of hollow organs such as blood vessels. However, all cells of the body have mechanosensitive channels (MSCs), including red cells. Most of these channels are cation selective and are activated by bilayer tension. There are also K^+ selective MSCs found commonly in neurons where they may be responsible for both general anesthesia and knockout punches in the boxing ring by hyperpolarizing neurons to reduce excitability. The cationic MSCs are typically inactive under normal mechanical stress, but open under pathologic stress. The channels are normally inactive because they are shielded from stress by the cytoskeleton. The cationic MSCs are specifically blocked by the externally applied peptide GsMtx4 (aka, AT-300). This is the first drug of its class and provides a new approach to many pathologies since it is nontoxic, non-immunogenic, stable in a biological environment and has a long pharmacokinetic lifetime. Pathologies involving excessive stress are common. They produce cardiac arrhythmias, contraction in stretched dystrophic muscle, xerocytotic and sickled red cells, *etc*. The channels seem to function primarily as "fire alarms", providing feedback to the cytoskeleton that a region of the bilayer is under excessive tension and needs reinforcing. The eukaryotic forms of MSCs have only been cloned in recent years and few people have experience working with them. "Newbies" need to become aware of the technology, potential artifacts, and the fundamentals of mechanics. The most difficult problem in studying MSCs is that the actual stimulus, the force applied to the channel, is not known. We don't have direct access to the channels themselves but only to larger regions of the membrane as seen in patches. Cortical forces are shared by the bilayer, the cytoskeleton and the extracellular matrix. How much of an applied stimulus reaches the channel is unknown. Furthermore, many of these channels exist in spatial domains where the forces within a domain are different from forces outside the domain, although we often hope they are proportional. This review is intended to be a guide for new investigators who want

to study mechanosensitive ion channels.

Key words: Channel; Mechanical; Patch; Force; Tension; Bilayer; Domain; Osmotic; Transduction; Biomechanics

Core tip: Mechanosensitive ion channels are found in all cells and their physiological function in most cells has yet to be defined inviting new researchers to the field. This review provides some guidelines to help newcomers understand key issues and potential artifacts.

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A WIMPY BACKGROUND

We are all familiar with many forms of mechanical transduction[1] including hearing[2], touch[3] and mechanical pain[4-7] that feed the central nervous system. In addition, there are the unconscious motor pathways bearing information about muscle stress and joint position[8,9]. Afferents in the autonomic nervous system service blood pressure regulation[8,10-12] and the distension of hollow organs[13,14]. While these reminders may be familiar to the readers of this journal, what may be less familiar is that all cells in our body, including red cells[15,16], are mechanically sensitive[17]. This cell sensitivity probably reflects our evolutionary origins[18]. Single cell prokaryotes like Paramecia have differentiated touch senses; if they bump into walls they back up, and if you bite their tails they run away[19-24]. We have the same heritage, but what are these sensors?

Mechanical sensitivity requires that forces do work on the sensor. This means that the sensors must be deformable, but that only narrows the field to all molecules since they can bend and stretch; it's a quantitative issue. Computational biology of molecular dynamics shows molecules moving under thermal and externally applied forces. How does one design a mechanical sensor? It's a matter of numbers. Sensors have a big output energy compared to the input energy. I will define mechanical sensors as those molecules or organelles in which the output energy, whatever that may be, is significantly larger than the input energy. Ion channels can do this because they are enzymes that dissipate lots of stored energy by catalyzing ion transport and their output energy is a function of the turnover number which can be 10^7.

All sensors have a background noise due to the random shaking of molecules (thermal fluctuations). In this review I will focus on ion channels as sensors (since that is my background), but all structural molecules can be viewed as mechanical sensors since they are compliant to applied force. The channels serve to couple mechanical stress to electrophysiology and biochemistry, typically by changes in calcium levels. This coupling of biochemistry to mechanics is familiar in muscle contraction, mechanically induced changes in gene expression, stem cell differentiation and changes in cell shape. We conjecture that all pathologies involve mechanics since they all involve a change in cell shape and that requires changes in force.

I will define mechanosensitive channels (MSCs), mechanically sensitive ion channels, as those channels whose dynamic range is fully accessible with physiologically relevant forces[25-37]. Many other ion channels, such as the voltage gated ion channels[38,39], are modulated by mechanical stress, but cannot span their dynamic range with mechanics alone. Many enzymes are also mechanically sensitive[40]. There are two basic types of MSCs: those gated by stress in attached structural proteins, and those gated by tension in the bilayer. MSCs in the differentiated sensory organs seem to be gated by forces in structural proteins[30,41-45] where the channel is in series with the extracellular matrix and the cytoskeleton (Figure 1). The other class of MSCs is gated by tension in the lipid bilayer (Figure 2)[24,42,46-50].

There is currently one drug known specific for MSCs of any kind, a peptide called GsMtx4 that is active on cation selective, bilayer-activated, MSCs[15,25,51-58]. Recently there has been a report of an organic molecule that tends to activate some of these channels[16].

This review is not intended to serve as a guide to the literature of MSCs, but addresses core issues that are required to understand how they work. For access to the general literature, there may be more review articles than research papers[18,24,42,46,47,59-63]! My primary goal is to familiarize newcomers to the field about what we have learned and to warn readers about some misleading dogmas to sensitize readers to critical interpretations of research papers. Many of the references in this paper come from the work in my lab, but that is not to say that they are the most important available, merely that I remember them better.

DEFINITION OF THE STIMULUS IN SIMPLE SYSTEMS

Lipid patch

One of the critical limitations of work on MSCs is that the stimulus tends to be imprecise. We can pull, indent, swell or shrink a cell, but what does the channel feel? We don't know, although but we can guess from the channel behavior. Even in plain lipid bilayer in a patch[46,47,61,64-66], we don't know the stresses with precision. Let's look at what might be considered the simplest of experimental systems: a patch of a liposome (Figure 3). Patches stick to the glass pipette. This adhesion produces a tension in the membrane that is a significant fraction of the lytic tension. This means that no one has ever recorded

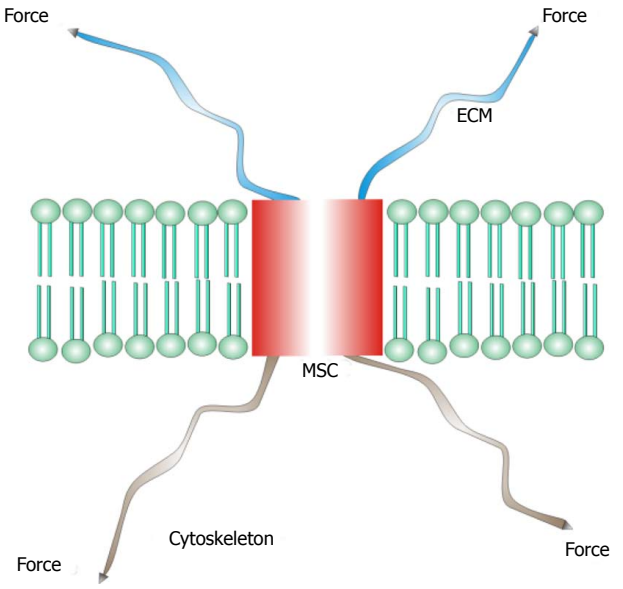

Figure 1 A mechanosensitive channel from a differentiated sensory organ where stress is passed through the channel from the extracellular matrix to the cytoskeleton. MSC: Mechanosensitive channel; ECM: Extracellular matrix.

patch currents of any kind except under extreme, nonphysiological, tension (there is one exception to this statement; we were able to measure MSC kinetics under low stress for short periods of time[67]). Patch tension is far in excess of anything seen in resting cells[31,33,68-70]. We know that tension can modulate many ion channels[38,39,67,71], probably transporters[72] and other membrane bound enzymes. When you read a paper about patch clamp recording, remember that all data was obtained under extreme membrane tension[59] and the patch data may not apply to the same channels *in situ*.

Returning to our ideal lipid patch, without pressure applied to the pipette the patch is pulled flat by adhesion to the glass. When we apply pressure, the patch bends, and the average tension in the membrane can be calculated from Laplace's law that states that the tension $T=Pr/2$ where P is the pressure across the membrane and r is the radius of curvature of the patch (not the radius of the pipette)[50,73-77]. From an image of a patch we should be able to calculate the membrane tension, and thus the stimulus that drives an embedded channel. Not so fast… notice that only the outer monolayer of the bilayer touches the glass. The inner monolayer is floating on the outer one[78-81], and as a fluid it cannot have any tension gradients at equilibrium[82,83].

Thus, even our simplest system has a gradient of tension normal to the membrane. You will notice that the papers on molecular dynamics simulations of MSCs apply a uniform tension across the bilayer in incorrectly compare those results to patch clamp data. While the mean tension in a membrane is related to the pressure across it by Laplace's law, models of membrane patches ignore the high membrane curvature where the patch contacts the glass (Figure 3) at the upper end of a seal[44,68,84,85].

Planar bilayer

We might imagine that an even simpler experiment that gets rid of the glass, a planar bilayer where the membrane is floating in space[66,86-90]. However, these bilayer lipids are attached to a support structure such as a hole in a Teflon partition. Lipids wet the support, and this adherence creates significant tension (approximately 5 mN/m) in the bilayer (the bulk lipid lining the hole in the support is known as the Plateau-Gibbs border[91,92], Figure 4). No one has made a measurement of channel activity in an unstressed planar bilayer[66,93], and for MSCs, this is a serious bias. The bilayer experiments of Coste *et al*[66] on the PIEZO channels suffer from this bias. If one is working with channels that inactivate, the resting tension in membranes, either in planar bilayers or patches, can put channels in an inactivated state making them invisible to a patch recording, and making a reconstitution experiment appear to have failed[18,43,48,94,95].

New method

There is one method, not currently in use, that might solve some of these problems. If the channels are reconstituted into large lipid vesicles (approximately 10 μm in diameter), and the vesicles are patched in "whole-cell-mode" then most of the membrane is not in contact with glass. By controlling the pressure in the vesicle which is large enough to measure accurately the radius of curvature, Laplace's law will provide reliable estimates of the mean tension and there won't be significant tension gradients.

STIMULATING CELLS

While lipid membranes are simple model systems, they are not cells. Cells are not homogenous solutions of macro molecules, but heterogeneous, anisotropic, viscoelastic/plastic structures made of multiple proteins and lipids with an extracellular matrix[43] that can carry stress between the exterior and the interior of the cells. But let's ignore these details for a moment and think of how we to experiment on cells.

Direct mechanical stimulation

The simplest stimulus is to poke a cell, usually with a fire polished pipette[93]. What does that do to MSCs? Imagine pressing a chopstick against a clump of Jello representing the cell interior. There is an indentation (more formally called a displacement) at the site of stimulation, but the amount of displacement decreases with distance from the site of stimulation. The stimulation is not uniform even at this macro level. This variation of stress/strain with distance occurs whether one stresses the cell with a pipette, a magnetic bead[96], a bead in a laser trap, an atomic force cantilever[97,98] or local perfusion[99]. It's

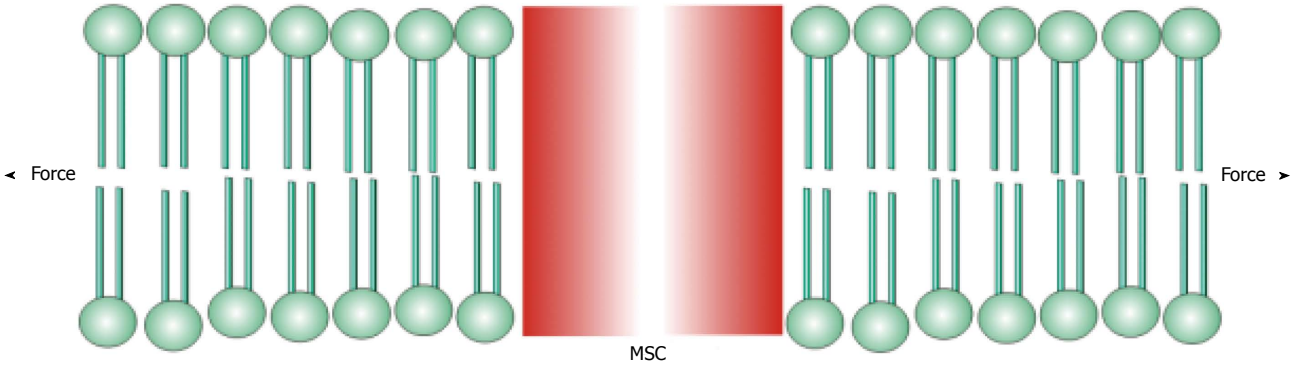

Figure 2 A mechanosensitive channel activated by tension (force) in the bilayer. MSC: Mechanosensitive channel.

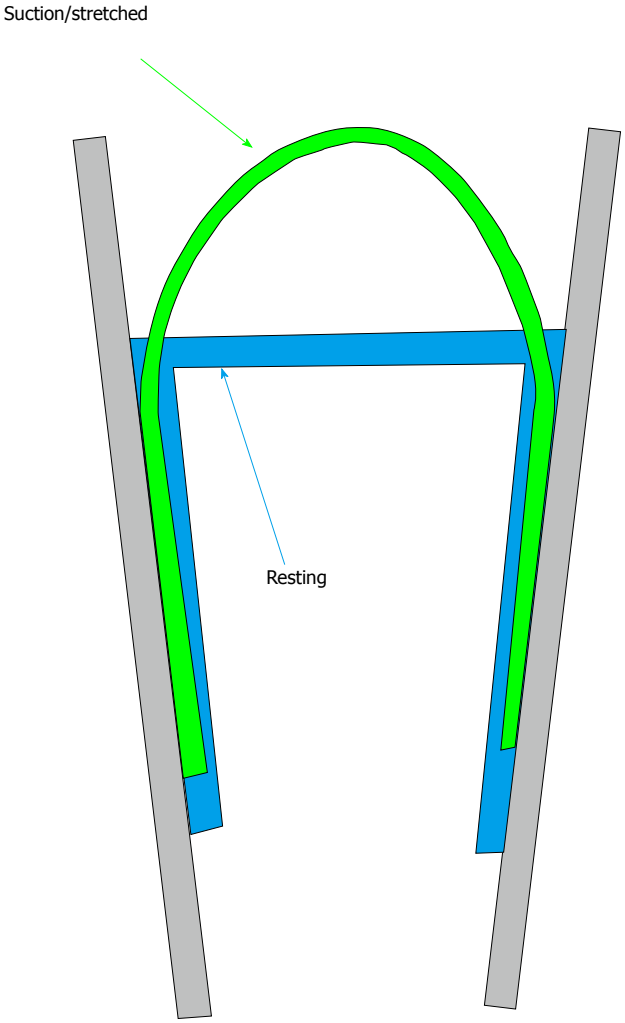

Figure 3 Cartoon of a patch pipette holding a lipid membrane at rest (pulled tight by adhesion to the glass) and under suction. Notice that suction peels the membrane off the glass a bit and functional channels.

just a property of a deformable material[100]. When you record mechanically-induced currents from a cell, the response is represents a mean value from a distributed stress, and it decreases nonlinearly with distance but covers larger and larger areas[100]. The best you can hope for is that the response is proportional to the stimulus, and hopefully monotonic. Further complicating

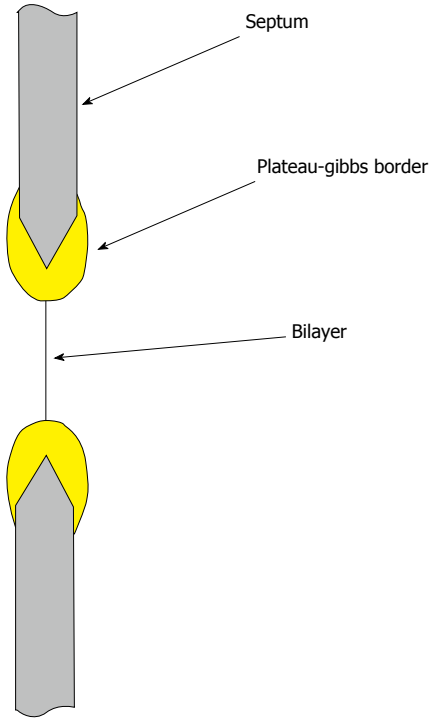

Figure 4 The planar bilayer is under tension, approximately 6 mN/m due to adhesion of the bulk lipid to the septum support.

those assumptions is the fact that the cell is viscoelastic and plastic - local stresses change with time even with a constant stimulus. Modelling of channels *in situ* usually assume the channels are uniformly distributed, but this is a bad assumption since, in general, they aren't (Figure 5). Remember to remain humble when interpreting your data since you really don't know the details of the stimulus.

In case I haven't yet scared you away from the field, let's look more closely at real cells. We will stick with whole-cell recordings where the local effect of the pipette glass is not significant. The key problem is determining what the channel feels. The ability to reconstitute bacterial MSCs in lipids shows the channels respond to tension in the bilayer and don't interact with a cytoskeleton, and that seems to apply to PIEZO MSCs as well[43]. What is the actual tension in the bilayer when

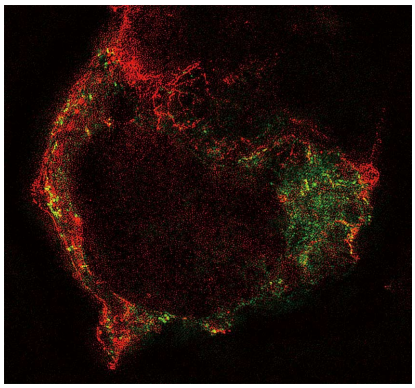

Figure 5 Structured illumination image of an human embryonic kidney cell cotransfected with human PIEZO1 channels labelled green and TREK-1 channels labelled red using green fluorescent protein mutants. Notice that the channels are in different structural domains and thus feel different forces. Notice also that TREK-1 tends to follow underlying cytoskeletal fibers. (Courtesy Gottlieb P).

MSCs are activated in cells? No one has measured it. The bilayer is supported by the cytoskeleton that shields the bilayer from excess stress (known as "mechano-protection"[36,101-103]). One experiment dealt with the distribution of stresses between the cytoskeleton and the bilayer in patches of an human embryonic kidney (HEK) cell, it there it was about 50:50. The cytoskeleton can thus alter the stress in the bilayer. Defects in the cytoskeleton can lead to diseases like muscular dystrophy[104-112].

Laser trap measurements of bilayer tension in resting cells suggests that it is negligible[69,70,113-115]. That fits our common observation that MSCs are not active in resting cells (Figure 6)[53]. Why do cells make MSCs if they can't be activated at normal stresses? Cortical stress is shared between the cytoskeleton and the bilayer, so bilayer stress reacts to cytoskeletal stress and *vice versa*, and these stresses are time dependent. The effective viscosity that makes responses time dependent arises from viscosity of the lipids and the dynamics of bonds in the cytoplasm[76,94,111,114,116-119]. The existence of connections between the bilayer and the cytoskeleton mean that any drugs that affect the cytoskeleton are likely to affect MSC activity, although drugs rarely are tested for these effects.

Adding to the complexity of defining the stimulus, lipids will flow under stress, and membrane lipids are not even homogeneous[46,76,116,120]. Spatial domains do exist[120-122] and physics tells us that the stress outside a domain is different from the stress inside a domain[73-75,123]. The energy gradient of stress at the edge of a domain is known as line tension. That affects the force inside the domain relative to that outside[75]. While we don't know in detail the stimulus at an MSC, we are safe in assuming the stress is greater than zero and less than the lytic limit of the bilayer.

If the transmembrane domains of a channel are thicker or thinner than the surrounding bilayer, the bilayer will bend at the boundary and those stresses are likely to modify MSC activity[124]. This is termed a hydrophobic mismatch, but the local curvature does not extend more than a few lipids from the channel[90,125-127]. However, amphipaths can dissolve in the membrane[128-131] and interact locally with the channel modifying the local stress and affecting channel gating. For example, the general anesthetics at clinically relevant concentrations cause opening of two-pore domain K^+ selective TREK-1 channels[132]. Opening these channels hyperpolarizes neurons possibly accounting for general anesthesia. The presence of these channels may explain why people can be knocked out by a blow to the head.

Osmotic stimulation

Suppose instead of these local mechanical stimuli we try for a more uniform stimulus like hypotonic stress? Cells swell with hypotonicity and we have been taught that swelling will stretch the membrane. If cells were spherical objects with a fluid cortex like red cells, that could work, but nucleated cells are filled with cross linked gels and the gels are what store most of the osmotic stress[133]. Consider the basic mechanics. Cells are not spherical so there are forces normal to the membrane. Secondly, with a given pressure across the membrane, the tension will depend upon the local radius of curvature (according to Laplace's law), and cells do not have uniform curvature. But a more serious problem is that nucleated cells have a cytoskeleton that acts like a sponge, a three dimensional object that fills the cell volume. The mechanics of three dimensional objects are different[134] from those of two dimensional objects like membranes[98].

We found that osmotic swelling doesn't make the membrane tense unless the cytoskeleton is disrupted[98], contrary to my intuition and years of textbook dogma. In fact, swelling tends to make cells softer[98]! How can that be? It turns out that everyone has done the experiment. When we pick up a dry kitchen sponge it is stiff. If we put it in the sink, it swells and soaks up water and it becomes softer. What is magic about a sponge? Nothing. It is just a set of cross-linked wettable polymers just like the cytoskeleton[26,135,136], and cells presumably can move water the same way without the need to move solutes. The cell membrane still remains the rate limiting step for water movement, but most of the energy from an osmotic gradient is in the cytoplasm and not in the membrane[133].

We visualized the distribution of osmotic stress in the cytoplasm using genetically coded optical stress sensors placed in structural proteins[26,27,29,30,119,137-139]. This three dimensional cross linked structure allows cells to withstand huge osmotic pressures[69]. (Ask yourself why sponges don't lyse.) Many cells, like bovine endothelial cells (BAECs), can withstand distilled water for hours and remain viable. The predicted osmotic (hydrostatic) pressure due to exposure of cells to distilled water is about 7 Atm, twice the pressure in a car tire. The cell' s stability under this huge gradient arises because the cell interior is glued together like a sponge. In the case of BAECs, this bonding allows the cells to face severe

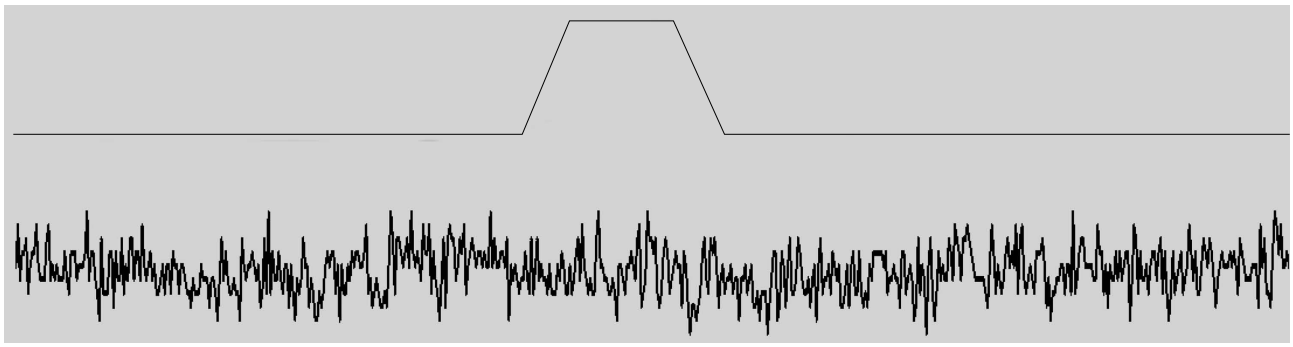

Figure 6 A whole-cell current recording (lower trace) from an human embryonic kidney cell in response to indentation of a few μm with a fire-polished micropipette in a piezoelectric manipulator (upper trace shows the cell indentation). There is no current associated with the indentation even though human PIEZO1 was cloned from the same cells! RNA expression does not mean the presence of functional channels, and even cells with no obvious endogenous currents may have channels, but they can't be activated because of mechanoprotection. They may become visible with large and/or repeated stimuli or treatment with agents like cytochalasin. (The trace is 1 s long and the RMS current noise is about 1 pA). RMS: Root-mean-square.

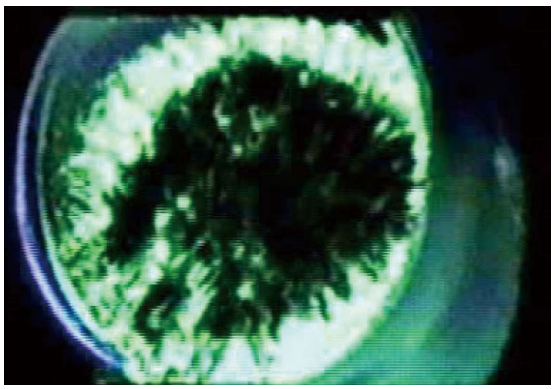

Figure 7 A frame from tomographic reconstruction of a patch of a Xenopus oocyte using high voltage electron microscope tomography[1]. The image shows cytoskeleton spanning the pipette and the bilayer is attached to the upper side but is not visible in this reconstruction due to its low density.

viscous drag in arteries where blood flow tries to rip the apical cortex from the basal cortex[27,140,141]. Osmotic stress does not stress the cell membrane very much, and despite many citations in the literature, osmotic stress should not be considered a "mechanical stimulus" of the cell membrane. Do not accept the results of papers that claim it is. Instead, treat those papers literally as dealing with the effects of osmotic stress.

There are a vast number of papers on cell volume regulation[55,98,142-147] invoking various ion channels such as the BK channels[148] and other K^+ channels[149], chloride channels[147,150-152] as well as neutral transporters[143,153,154] and water transporters[155-157] as well as the cytoskeleton[26-29,98,139,158] and host of calcium and other intracellular messengers[15,159-161]. Given the vast scale of modulators and potential effectors, it is unwise to think of cell volume as a specific stimulus.

Patch clamp stimulation

We all know about patch clamp recording and the revolution it created in our understanding of ion channels[162,163]. But what is a patch? The dogma says it is a bilayer containing channels[163] that spans the pipette. However, unless you are working with lipid vesicles, that is incorrect; patches are pieces of the cell cortex. Microscopy of patches (light microscopy[44,164] and electron microscopy[1,165-168]) show that patches are samples of the cell cortex, including the cytoskeleton (Figure 7) [1,165].

Whenever you make a patch, cell-attached or excised, the bilayer that contains your channels shares its stress with the cytoskeleton. How much does the bilayer feel in this composite structure? In the only published paper on the matter[169], we compared the amount of mechanical stress required to break a patch (pipette suction) with the voltage required to break the patch (typical of patch clamp "zap" voltages). The mechanical stress measured the lytic stress of the entire cortex. Voltage measured only the stress of the bilayer since that is where the voltage drop occurs. We measured the voltage required to lyse a patch as a function of the mechanical stress; the more mechanical stress, the less voltage. Since voltage only exerts force on the bilayer, we could separate the bilayer stress from the mean stress. It turns out that the bilayer lyses with a constant energy density, whether it comes from mechanics or voltage. For our particular cells, HEK-293, about half of the applied stress was in the bilayer and the rest in the cytoskeleton, but that result is from patches and we do not know how that applies to resting cells.

Regardless of the degree of stress sharing, no one has ever measured channel currents in a patch that emulates the tension characteristic of a resting cell[50,76]. The magnitude of the resting stress in a patch was emphasized to us when we tried to use used Triton-X100 to lyse patches. It doesn't work. The patches are stable. The reason is that detergents work by forming micelles. If you want to form a micelle in a patch under tension, you need to increase the membrane area since a plane plus a sphere has more area than the plane. The energy required to change the area of a membrane under tension T is $\triangle G = T\triangle A$ where $\triangle A$ is the change in area (Hooke's law in two dimensions). The energy available to the detergent is insufficient to form a micelle, but the

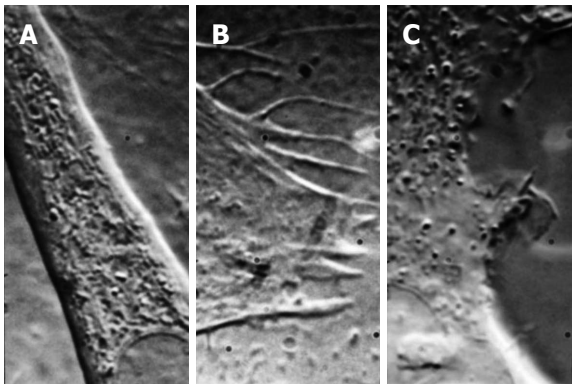

Figure 8 Differential interference contrast images of three different cell types (mouse myotube, left; rat astrocyte, center, human embryonic kidney cell, right) showing the variability of membrane structure and how patch clamp recordings are expected to be variable. The structures above change rapidly over time (this is a frame from a movie, Courtesy Suchyna T).

same detergent works well in the resting cell the patch because its membrane is not under tension.

Is the cytoskeleton of a patch the same as that in a cell? We don't know, but we do know that the chemical composition of a patch is different from that of the cell it was taken from[44]. We labelled different components of cell membranes and then patched them. We found that some elements made it to the pipette-spanning dome, and some didn't, notoriously the extracellular matrix[44]. That never even made it into the seal region. Some ion channels made it, and some didn't. You need to think of the pipette as a silica column. Biochemists know proteins stick to silica, so after dragging a membrane up a pipette, some things will stick to the glass and get filtered out, and some will make it to the dome.

The heterogeneity of the patch emulates the heterogeneity of the cell membrane and we know that the cell membrane is not homogenous (Figure 8). Even pure bilayers may not be homogeneous. Like ice and water, there are phase separations[170]. The amount of each phase (the fraction of total membrane area) is modulated by internal and external conditions. Cell membranes are much more complicated. If you look at a time lapse movie of cells, you will be impressed by the motion of the cell surface. Imagine your patch pipette coming down on one of these cells and then try to figure out which piece of membrane you patched. The answer, of course, is that you have no idea. Furthermore, given the data showing changes in patch composition with patch formation[44], and visible domains in a patch, you are in fact recording from a new mixture of cellular components. We suspect that patches might contain membrane from the endoplasmic reticulum and other organelles as well as the plasmalemma. Someone needs to check on that. We think that patch clamp recordings are as reproducible as they are because the formation of the patch helps to homogenize the components. In any case, be cautious about assuming that the properties of currents you get from a patch are the same as you would observe *in situ*.

WHAT CHANNEL ARE YOU RECORDING FROM?

We know that most if not all types of cells have endogenous cationic MSCs[15,17,51,55,128,171-174]. You may not see them frequently as they are normally closed because bilayer stress is shielded by the cytoskeleton ("mechanoprotection")[71,93]. You must know your background channel activity if you want to examine cells containing transfected channels. Treating the cells with cytochalasin or latrunculin to break up the cytoskeleton will reduce mechanoprotection and make background channels more visible[171]. Cell lines vary from lab to lab. According to the literature Coste *et al*[93] used N2A cells to clone PIEZO1, but Lee *et al*[71] used the same cell line and found no background PIEZO1 and 2 activity. Why? I expect that the cytoskeleton changes with passage number and with different batches of serum.

Because of the nearly universal presence of background channels, seeing a cationic MSC current after transfection does not mean you are seeing the channel coded by the DNA you used to transfect the cells. Furthermore, the expression of an MSC (or probably many other proteins) can cause massive structural changes in the cytoskeleton, even if the channel is non-conducting[175]. Thus, the process of transfection alone (not the effect of the transfection reagents, *per se*) can modify the forces that reach the channels.

We can now study cytoskeletal protein stress using genetically coded stress sensors[3,25,26,28-30,119,137-139,176]. The same issues apply to siRNA since suppression of one protein can affect others. For example, we showed that cytochalasin or colchicine affects the stress in actinin, spectrin and filamin and likely other structural proteins that are not judged to be the drug targets. When we modify any protein in a cell, we modify the stresses in the elements that are coupled to that protein.

Transfection can be a dangerous game. You can easily show modified RNA expression, we know that RNA expression is not cleanly related to the presence of functional channels. We cloned the human form of PIEZO1 and 2 from HEK cells[53], a human cell line of neural origin that usually exhibits little background MSC activity (Figure 6). The N2A cells that Coste *et al*[66] initially used to isolate PIEZO1, had no background MSC activity in other samples of the same cell line[71]. So how do we know what channels produced the currents we are looking at?

The best test would be to create a mutant channel with similar gating functions but with visibly different ionic selectivity than the endogenous channels. You cannot depend upon channel conductance alone[57] as a sound marker of expressed channels since it is easy to find situations in which the environment: cell-attached patch, inside-out or outside-out patch, or whole-cell, or planar bilayer have different conductances[66,177]. You would want a channel with big differences in selectivity, ideally a change from cation to anion selectivity!

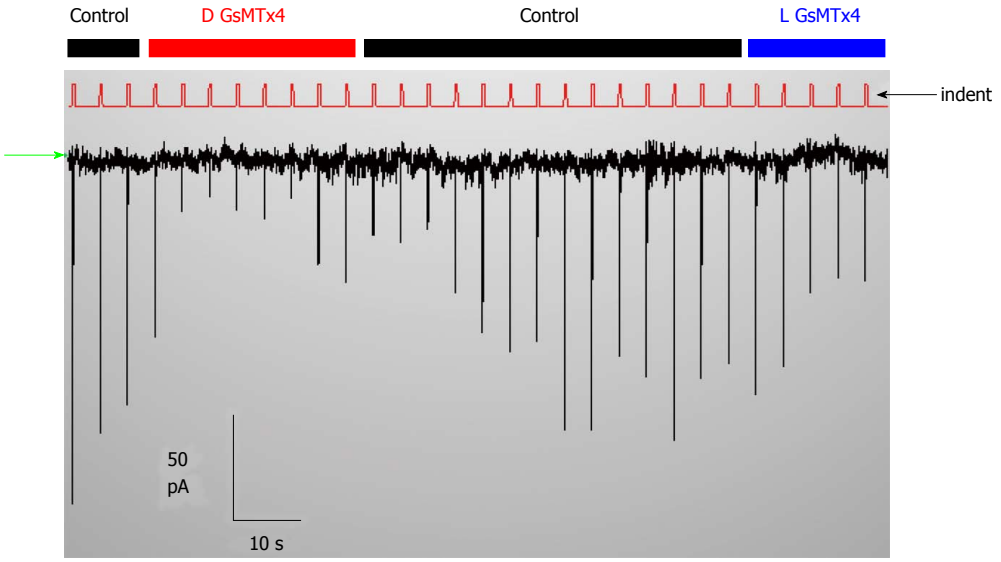

Figure 9 There are no PIEZO1 currents in this resting whole-cell recording unless the cell are indented (the red trace labelled indent shows the stimuli). The inhibitor GsMtx4 is effective in suppressing the stimulus-induced current but causes no change in the holding current. The stimuli are activated by computer control (www.qub.buffalo.edu). The baseline current (green arrow at left) is not affected by adding the D or the L enantiomers of GsMtx4, but the currents that are evoked by indentation are inhibited. Thus, tension in the resting cell is insufficient to activate PIEZO1.

You also cannot trust the channel kinetics as a marker since the kinetics of the channels depend upon their environment[177,178] and you have little control of that. Furthermore, you do not know if the channel you are trying to express might have a subunit that associates with an endogenous channel subunit or accessory protein, or induces the expression of previously unexpressed endogenous protein. It is well known that mechanical stress alters gene expression[179-182]. We know that expression of two different MSCs can create currents that do not belong to expression of either one alone[71]. There are no clean solutions in cells. We arbitrarily tend to look more closely at transfections that produce currents much larger than the background channels. We also don't know that we are looking at homomers of the transfected proteins, but we often make the simplifying assumption they are (this also aids in grant funding). Expression of green fluorescent protein labelled channels on the cell surface[43,48] or in patch membranes[44] does not mean that the fluorescent object is a functional channel, simply that the protein is present. We have tested the mechanical sensitivity of labelled transient receptor potential-canonical channels in patches and found they are present but are not mechanically sensitive[183].

WHY BOTHER WITH MSCS?

Cationic MSCs are normally protected from cell stress, so what do they do for a living? They do not seem to participate in behavior of normal hearts[184], but they do in stretched hearts where they seem to play a role in generating arrhythmias like atrial fibrillation[184]. They also play a role in muscular dystrophy where the channels produce a Ca^{2+} leak when the muscle is stretched[110,185]. We have come to believe that the typical cationic MSCs, like PIEZO1, serve primarily as sensors for potential bilayer failure. They would inform the cytoskeleton that the local bilayer is under excessive stress and likely to break, and the ion fluxes through the channel are signaling for mechanical reinforcement. The channels are functioning a bit like fire alarms whose function you unaware of until disaster looms. If the channels are closed in the resting cell you will not see the effect of inhibitory drugs like GsMtx4 on the currents (Figure 9). But if the same drug is active on open MSCs, you will see an effect, but to open the channels may require pathologic stress. Since channels like PIEZO inactivate, the information about the excess stress is transitory.

PIEZO1 mutations can cause anemias[43], and we have wondered why are these channels that inactivate quickly (< 30 ms) are present in red cells[186]. When does a red cell need such a short lived channel? We guess that the only time red cell stress becomes "pathological" is upon entry and exit from a capillary or perhaps a bifurcation. It might modify ion and water concentrations to reduce stress on the membrane as it is highly deformed upon entering the capillary. The same channels may be involved in sickle cell anemia where hemoglobin crystals push out on the membrane activating PIEZO1[15].

The universal presence of MSCs fits the common demand of all cells to avoid lysis and that occurs in disease. GsMtx4[187] and other agents that might act specifically on MSCs promise to be a new class of therapeutic agent with ideal selectivity they would only affect sick cells. We have found that GsMtx4 can be administered to mice daily for a month with no effect on behavior, and it can be injected into the CNS with no effect on behavior, but it does work to inhibit volume stimulated arrhythmias[188] and the phenotype of muscular dystrophy[109].

There is evidence that PIEZO2 channels may serve

a sensory role in nociception[4]. Since PIEZO was only cloned a few years ago, we have a lot more work to do. A nagging problem is why is PIEZO so big[189] - it is the largest transmembrane protein (approximately 2500 amino acids) and even tends to form tetramers with a MW of about 10^6 with the N and C termini about 20-30 nm apart[190] making us suspicious that PIEZOs have other functions; a large size is not necessary for MSC function[43,67]. There are many kinds of MSCs[191-194], nearly a dozen in bacteria alone, so we have lots of interesting problems to keep us busy.

CONCLUSION

This review has two goals, nominally for investigators new to the field of mechanotransduction: (1) Be humble about your data because you generally don't know your stimulus, and be explicit about your assumptions so people can read your paper properly. Quantitative models of the data have the intrinsic appeal of making the assumptions explicit; and (2) Create new preparations that can answer some of the pressing host of new questions.

ACKNOWLEDGMENTS

This paper represents several decades of work and the contribution of dozens of people, too many to name here. Much of the data and the ideas in recent years come from Thomas Suchyna, Philip Gottlieb, Fanjie Meng, Chilman Bae, Radhakrishnan Gnanasambandam, Arthur Beyder, Chiara Spagnoli and Susan Hua. You can find many of the other collaborators in the cited papers.

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REVIEW

Molecular pathogenesis of glioblastoma multiforme: Nuances, obstacles, and implications for treatment

Siddharth K Joshi, Nevena Lucic, Richard Zuniga

Siddharth K Joshi, Department of Medicine, New York Methodist Hospital, Brooklyn, NY 11215, United States

Nevena Lucic, Richard Zuniga, Division of Hematology/Oncology, Department of Medicine, New York Methodist Hospital, Brooklyn, NY 11215, United States

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Correspondence to: Richard Zuniga, MD, Division of Hematology/Oncology, Department of Medicine, New York Methodist Hospital, 506 6th Street, Brooklyn, NY 11215, United States. rickzunigaperu@hotmail.com
Telephone: +1-718-2081820
Fax: +1-718-2081822

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Abstract

Glioblastoma multiforme (GBM), the literal apogee on the hierarchy of malignant brain tumors, remains one of the greatest therapeutic challenges in oncology and medicine. Historically this may be contextualized in the fact that the medical and scientific communities have had a very elementary understanding of its intricate and complex pathophysiology. The last 10-15 years have yielded a number of studies that have elucidated much of the molecular and genetic complexities of GBM that underlie its pathogenesis. Excitingly, some of these discovered genetic mutations and molecular profiles in GBM have demonstrated value in prognostication and utility in predicting response to treatment. Despite this, however, treatment options for patients have remained somewhat limited. These treatment options are expected to expand with the availability of new data and with the transition of novel treatment modalities from animal to human studies. This paper will have a threefold objective: provide an overview of the traditional paradigm in understanding and treating GBM, describe recent discoveries in the molecular pathogenesis of GBM against this historical backdrop, and acquaint the reader with new treatment modalities that hold significant therapeutic potential for patients.

Key words: Molecular pathogenesis; Temozolomide; Glioblastoma multiforme; Treatment resistance; Hypoxia; Recurrent glioblastoma multiforme; Bevacizumab

Core tip: This paper provides the reader with an overview some of the primary molecular markers that are implicated in the pathogenesis glioblastoma multiforme (GBM). It provides a robust review of the evidence that supports the use of these molecular markers for both prognostication and prediction for response to treatment. It gives the reader context for understanding the hypoxia model and how it informs treatment resistance in GBM. It provides an overview of cancer stem cells and their role in GBM biology. And it acquaints the reader with a few of the new, promising treatment modalities that are emerging.

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INTRODUCTION

Glioblastoma multiforme (GBM) belongs to a class of brain tumors known as gliomas, so named because they arise from glial cells (astrocytes, oligodendrocytes, ependymal and schwann cells). Glial cells have traditionally been understood as the workhorse cells of the central nervous system (CNS), providing the needful nutrients, oxygen and stromal support for neural cells. Recent studies have shown glial cells to have a more central and independent role in the CNS than historically thought, acting alongside neural cells in neurotransmission[1].

Since 1979, gliomas have been classified by the World Health Organization into 4 classes based upon histopathology, each successive class exemplifying features more consistent with malignancy[2] (Table 1). Indeed, of the 4 classes, only grades Ⅲ and Ⅳ are considered malignant gliomas due to possessing telltale histological features (increased cellularity, abnormally increased mitotic activity, nuclear atypia). On this hierarchy, GBM is classified as grade Ⅳ due to the typifying unique characteristics of ubiquitous neovascularization and dramatic necrosis of neoplastic tissue (due to the extent of cell turnover).

Epidemiology

GBM is the most common malignant brain tumor, and histologically is second in incidence only to meningiomas when considering all intracranial neoplasms, both malignant and benign. Based upon data compiled by the Central Brain Tumor Registry of the United States Statistical Report, GBM makes up 15.6% of all brain tumors and 45.2% of primary malignant brain tumors[3]. The incidence of GBM increases with age; highest rates are observed in 75-84 years old and, conversely, comprise only about 3% of brain and CNS tumors in 0-19 year olds. For reasons unclear, GBM is slightly more common in males.

Primary vs secondary GBM

GBM may be classified as either primary or secondary. As connoted by the names, primary GBM comes from native, wild-type glial tissue whereas secondary GBM comes about through malignant changes in lower grade gliomas (Grades Ⅰ and Ⅱ). When a case of GBM is diagnosed, determining whether it is primary or secondary is germane to the clinician for it allows him/her to make initial informed impressions on the biological and clinical behavior of the tumor, provides utility in prognostication and, increasingly, is guiding clinicians in predicting responses to molecular/targeted therapies. Before proceeding to characterize key genotypic differences between primary and secondary GBM, it is of interest to briefly delineate defining epidemiological and clinical features of these respective categories. Upwards of 90% of GBM cases are primary. These tumors are afflictions of the elderly, the mean age at diagnosis being 62. And they carry with them a uniformly poor prognosis at the present time, with roughly two-thirds of patients dying less than 3 mo from the time of diagnosis[4].

Secondary GBMs, by contrast, are predominantly cancers of a younger population, the mean age at diagnosis being 45. This tumor is characterized by a more indolent time course than Primary GBM, progressing from lower grade gliomas over the course of years as opposed to months[4]. Indeed, a population-based study from 2005 reported a mean time of 5.3 years to be the amount of time it took for low-grade astrocytoma to develop into GBM. In the case of anaplastic astrocytoma the mean time reported was 1.4 years[5]. Secondary GBMs represent a small minority of cases, accounting for less than 10% of total GBMs.

Primary GBMs have trademark molecular abnormalities that distinguish them from secondary GBMs, and it is these unique genetic aberrations that give each class the distinct characteristics discussed above (Figure 1). These are: mutations in the gene encoding the epidermal growth factor receptor (EGFR) protein that result in its amplification, loss of heterozygosity (LOH) of Chromosome 10q, phosphatase and tensin homolog (PTEN) deletion on Chromosome 10, and p16 deletion. Conversely, in Secondary GBMs, mutations of the ubiquitous p53 oncogene and of the gene encoding the platelet-derived growth factor receptor (PDGFR) protein are culpable for malignant transformation of lower grade gliomas[6]. A few of these molecular anomalies will be treated in detail in the paragraphs to follow (Tables 2 and 3).

Many of the gene products inextricably involved in the development of GBM are growth factor signal transduction proteins that transduce an extracellular signal *via* ligand binding into a cellular response. The cellular response regulated by these proteins is proliferation and growth. A very carefully orchestrated combination of positive and negative regulatory ligands in the extracellular milieu ensures that in the normal homeostatic state, growth and proliferation of glial cells is kept in check. A common recurring theme in malignant transformation is mutations that cause amplification or overexpression of the signal transduction protein products.

One of the best characterized signal transduction proteins that brings about malignancy in more than 40% of cases of primary GBMs is EGFR[4]. Among tyrosine receptor kinases, EGFR belongs to the ErbB receptor family, bearing significant genetic homology to three others-HER2/c-neu (ErbB-2), Her3 (ErbB-3) and Her4 (ErbB-4). The wild type function of EGFR is contingent upon binding a specific extracellular ligand

Table 1 World Health Organization classification of gliomas

Localized astrocytoma
WHO grade Ⅰ
Pilocytic astrocytoma
Pleomorphic xanthoastrocytoma
Subependymal giant cell astrocytoma
Diffuse astrocytomas/oligodendrogliomas
WHO grade Ⅱ (Astrocytoma)
Fibrillary
Protoplasmic
Gemistocytic
WHO grade Ⅱ (Oligodendroglioma)
WHO grade Ⅲ (Anaplastic astrocytoma)
WHO grade Ⅲ (Anaplastic oligodendroglioma)
WHO grade Ⅳ (Glioblastoma multiforme)
Giant cell glioblastoma
Gliosarcoma

WHO: World Health Organization.

Table 2 Primary glioblastoma multiforme *vs* secondary glioblastoma multiforme

	Primary GBM	Secondary GBM
Mean age at diagnosis	Approximately 62 yr of age	Approximately 45 yr of age
Percentage of cases	> 90%	< 10%
Clinical course	Rapid	Smoldering
Origin	*De novo*	Grade Ⅱ / Ⅲ astrocytomas

GBM: Glioblastoma multiforme.

Table 3 Hallmark genetic mutations, primary glioblastoma multiforme *vs* secondary glioblastoma multiforme

Primary GBM	Secondary GBM
EGFR overexpression/amplification	PDGFR overexpression
Loss of heterozygosity of Ch. 10q	Loss of heterozygosity of Ch. 10q
PTEN deletion on Ch. 10	p53 mutations
p16 deletion	p16/Rb pathway aberrancies

GBM: Glioblastoma multiforme; EGFR: Epidermal growth factor receptor; PDGFR: Platelet-derived growth factor receptor; PTEN: Phosphatase and tensin homolog.

at its extracellular domain, ensuring that it remains coordinated with physiological needs. On binding the ligand, inactive monomers of EGFR dimerize to an active form and provoke autophosphorylateion of the intracellular, C-terminal domain at multiple tyrosine residues. Certain intracellular signaling proteins bind EGFR and concomitantly activate signal transduction cascades. The end result is increased expression of genes that are involved in a pro-growth phenotype.

When mutated in GBM as well as other malignancies, the *EGFR* gene is typically amplified, in which case the protein is autophosphorylated constitutively and is thereby overactive.

However, in GBM there exists a unique mutation that generates a mutant protein Epidermal growth factor receptor variant Ⅲ (EGFRvⅢ) which is over-expressed. The mutation in the gene - a deletion of exons 2-7 -causes a deletion in the extracellular domain of the EGFRvⅢ protein that makes it inaccessible to extracellular regulatory ligands. This in turn leaves the protein in a constitutively active state that begets a slew of malignancy-specific features: cellular proliferation, the ability to invade other tissues, angiogenesis, and abnegation of the normal process of apoptosis. An interesting feature from the treatment perspective is that the deletion that yields the EGFRvⅢ protein encodes a codon that is not found in wild-type DNA and is unique to GBM[7]. Thus, conceivably, this sequence can be pursued as a specific molecular target in next generation treatment. In fact, studies are underway seeking to target EGFRvⅢ as a target. The phase Ⅲ ACT Ⅳ trial underway is investigating the cancer vaccine rindopepimut for this very purpose[8].

TREATMENT

Surgery

Whereupon magnetic resonance imaging all but cinches the diagnosis, the gold standard for confirmation remains tissue biopsy. Though this may be accomplished by stereotactic brain biopsy alone, tissue is more commonly procured with maximally safe surgical resection. As it stands, maximally safe surgical resection is by no means curative as by the time of diagnosis, the tumor has invariably insinuated itself deep into vital, surgically inaccessible tissue. This said, rote surgical resection does still provide the patient with relief from symptoms wrought by mass effect of the tumor. There is also demonstrable improvement in survival by resection of tumor burden, albeit marginal[6].

Radiation

Adjuvant radiotherapy has been an established cornerstone in the treatment of GBM since 1979, when publication of the seminal study by Walker *et al*[9] showed that patients treated with radiation showed longer survival than those treated with best supportive care.

A major obstacle in the radiotherapy of GBM is the problem of radiation resistance, which is recurrence of the tumor within the high dose region[7]. The existence of this phenomenon implies that the amount of radiation administered (and as tolerated without excessive toxicity) is not enough to eradicate in entirety tumor existing in the radiation field. It is hypothesized that some of the hallmark genetic mutations characteristic of GBM contribute to the phenomenon of radiation resistance. Studies that have looked at increasing the dose of radiation to the limit tolerated-up to 90 Gy-have not demonstrated a discernible benefit of this strategy.

Having discussed the limitations of current radiotherapy, it begs discussing new modalities being investigated that intend to overcome these limitations

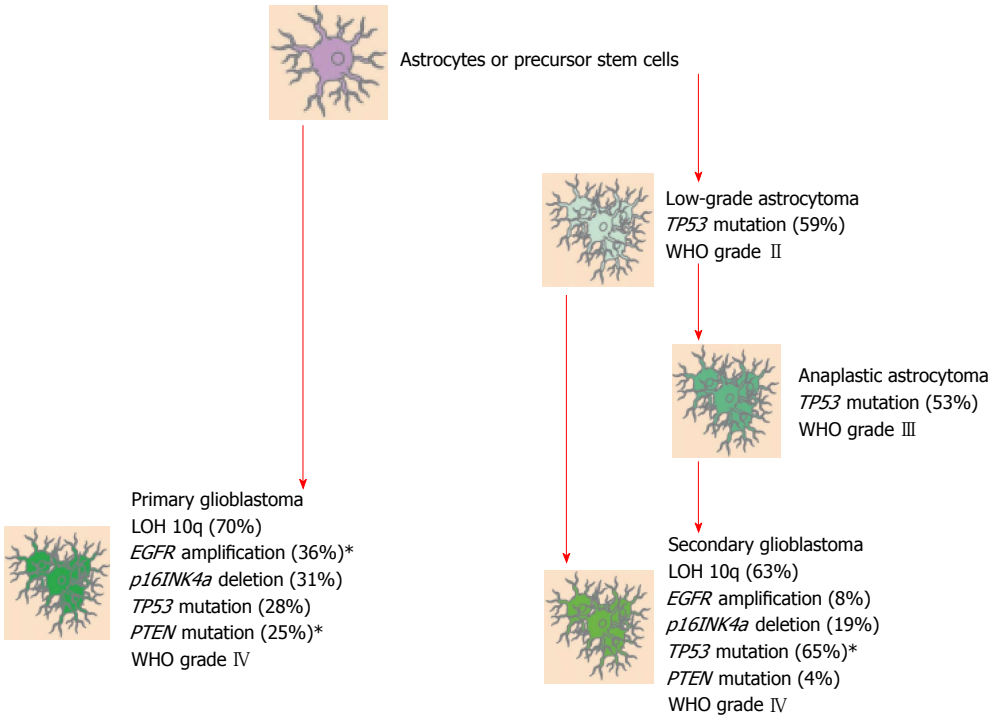

Figure 1 Genetic mutations pathways implicated in the development of malignant gliomas (76). WHO: World Health Organization; LOH: Loss of heterozygosity; EGFR: Epidermal growth factor receptor; *PTEN*: Phosphatase and tensin homolog.

which will be discussed later.

Chemotherapy

The chemotherapeutic agent temozolomide has been available since 1999. Pharmacokinetically, temozolomide is an oral agent with effective absorption and excellent bioavailability. Temozolomide is metabolized into 5-(3-methyltriazen-1-yl) imidazol-4-carboxamid. The therapeutic potential of temozolomide lies in the alkylation/methylation of the DNA of tumor cells, typically occurring at the N-7 or O-6 positions of guanine residues. Methylation causes irreversible DNA damage which in turn provokes tumor cell death[10,11].

The use of temozolomide as standard of care adjuvant chemotherapy for GBM patients is largely the result of a seminal trial by Stupp *et al*[12] in 2005. This randomized controlled trial compared the use of irradiation alone to the use of concurrent radiation and temozolomide chemotherapy followed by 6 cycles of adjuvant temozolomide. In the experimental group, concurrent temozolomide was administered at 75 mg/m^2 daily during irradiation (both arms received 30 fractions with total dose of 5500 to 6000 cGy) followed by 6 cycles of temozolomide 150 mg/m^2 (days 1 to 5 of cycle 1) then 200 mg/m^2 on days 1 to 5 of cycles 2-6, repeated on day 29.

It was found that temozolomide improved median overall survival (OS) (14.6 mo *vs* 12.1 mo), 2 year OS (27.2% *vs* 10.9%), 3 year OS (16.0% *vs* 4.4%), and 5 year OS (9.8% *vs* 1.9%). These statistically significant results corroborated the superiority of temozolomide and that continuously improved over time.

MOLECULAR MARKERS WITH CLINICAL RELEVANCE IN GBM

MGMT and chemotherapy resistance

It is of importance to reconcile the positive results of temozolomide use in GBM treatment with the fact that, as discussed at the beginning of this paper, the overall outcomes in patients with GBM still remain unequivocally poor. One important concept that helps to explain this in part is chemotherapy resistance and in the case of temozolomide, through damaged DNA repair.

These chemotherapy resistant cells express a protein, O6-alkylguanine DNA alkyltransferase (AGT), encoded in humans by the O-6-methylguanine-DNA methyltransferase (*MGMT*) gene. The AGT protein removes the alkylated moiety on the O6 position of guanine and renders the therapeutic modality of temozolomide obsolete. It has been found that there exists an epigenetic variant of tumor cells that are able to circumvent this mechanism of chemotherapy resistance. These tumor cells possess a protein that is responsible for methylation of the MGMT promoter; this methylation serves to silence the *MGMT* gene. As a result, such tumors are thought to be more sensitive to temozolomide. Based upon this, this molecular marker for MGMT methylation has been investigated as a means of predicting response to treatment with temozolomide. As early as 2005, the group of Stupp *et al*[13] recognized the implications of this gene in therapy and conducted a retrospective analysis on the tumors culled from subjects in their pivotal study establishing superiority

of adjuvant chemotherapy and radiation to radiation alone. For 203 patients whose tumors were found to possess the *MGMT* methylation gene, a substantive difference was found, with the progression free survival (PFS) being substantially greater in the experimental arm receiving temozolomide with radiation therapy (RT) than the control group receiving RT alone. On the basis of these findings, the prevailing thought was that possession of the *MGMT* methylation gene predicted favorable response to treatment with temozolomide. This premise of using the *MGMT* methylation gene for prediction of response to treatment with temozolomide was challenged in 2011 by the RTOG 0525 study[14]. The purpose of this study was to look at a proposed strategy for overcoming acquired temozolomide resistance, specifically whether there was a survival difference between the use of standard schedule of temozolomide or an altered schedule in which the same total dose of temozolomide was delivered in higher fractions, allowing for a 3 wk on, 1 wk off dosing. When these patients were stratified based on MGMT status, there was an OS of 23.2 mo in patients with tumors possessing the *MGMT* methylation gene *vs* 16 mo in those harboring unmethylated MGMT status. Thusly, the current paradigm is that possession of the *MGMT* methylation gene prognostically bodes better for patients receiving standard adjuvant treatment than those that do not possess in the general population but does not necessarily predict response to treatment with temozolomide. An important demographic caveat exists, however. It was found on that basis of multiple studies that the *MGMT* methylation gene does predict favorable responses in terms of survival benefit in elderly patients (age greater than 70) with GBM who receive TMZ and radiation *vs* RT alone[15-17]. This is important in that uses of different modalities of treatment necessarily must be used more conservatively and sparingly in elderly patients who have more limited physiological reserve with which to contend with the ill effects of such treatments.

Interestingly, there is another more nuanced twist to the MGMT story. Recent studies have revealed that in some tumors, the *MGMT* gene-and resistance to TMZ--is effectively silenced even without possession of the *MGMT* methylation gene. What these studies have found is that *MGMT* expression is also post-transcriptionally regulated by micro-RNAs[18]. MicroRNAs (miRNAs) are non-encoding RNA molecules 20-23 nucleotides in length that inhibit the translation and stability of messenger RNA (mRNA). MicroRNAs have a potent presence in the regulation of post-transcriptional gene expression as they "flag" mRNAs which leads to their decay and influences essential cell functions, *i.e.*, replication, proliferation, metabolism, programmed cell death, *etc*.[19]. Low *MGMT* expression in promoter unmethylated tumors was found to be due in part to the expression of miR-181d, a miRNA that suppresses *MGMT* expression. There have been additional micro-RNAs identified that bind directly to the MGMT 3' UTR and purportedly result in loss of MGMT protein expression both in pre-clinical and clinical studies.

Deletion, mutation and LOH on chromosome 10

There are a number of genes on chromosome 10 of which mutation, deletion or LOH has been established in the development of GBM malignancy. These will be considered in turn.

A well-described phenomenon engendering tumorigenesis is LOH. In somatic cells, many tumor suppressor genes bear heterozygosity by merit of having inherited unique single nucleotide polymorphisms in different regions in that gene. Thusly, one allele in the pair for that gene is different from the other. In the process of LOH, a portion of or a complete chromosome in a diploid pair is deleted. If this portion contains a tumor suppressor gene, then the cell containing that deletion exhibits LOH for that gene or chromosome. When the remaining copy of the tumor suppressor gene incurs a mutation, the cell is no longer protected by that tumor suppressor gene and the biology of malignancy is begotten.

The LOH phenomenon specifically involving alleles of tumor suppressor genes in parts or all of chromosome 10q has reliably been demonstrated in the molecular pathogenesis of GBM[20]. A specific example is allelic deletion of the phosphatase and tensin homolog gene, or PTEN, located at locus 10q23[21] (Figure 2).

The wild type *PTEN* gene is a tumor suppressor. The product of this gene is involved in many different signaling pathways in its capacity as a phosphatase. The most important of these pathways is the PI3K/Akt pathway[21]. When an extracellular ligand binds to its correspondent receptor, *e.g.*, EGFR, HER2, IGFR, the protein PI3K is activated and creates PIP13. PIP13 in turn recruits the Akt to the intracellular surface of the cell membrane and subsequently activates the PI3K/Akt pathway. Activity is positively regulated by the PIP3 gene product. This pathway promotes a number of progrowth phenotypes, including cell cycle progression, protein synthesis, inhibition of apoptosis and cell migration. When PIP3 is dephosphorylated by PTEN to PIP2-a, the PI3K/Akt pathway is downregulated and antagonizes the progrowth phenotype. Thusly, when PTEN activity is lost through mutation or LOH, PIP3 accumulates and begets malignant growth though constitutive activation of the PI3K/Akt pathway. Mutations in *PTEN* have been implicated in a variety of malignancies, including prostate, gyn malignancies, breast, pancreatic, melanoma and GBM[21].

PTEN LOH mediating malignant features in GBM has been found to occur in as much as 60%-80% of all cases[21]. Historically, studies concerning LOH or mutation in *PTEN* had proposed a value in prognostication, *i.e.*, that loss of PTEN would make for a poorer prognosis[21]. This was particularly so prior to acceptance of TMZ as standard adjuvant treatment of GBM. However, a recent study out of Cedars-Sinai medical center appears to refute this understanding[22]. Indeed, the premise

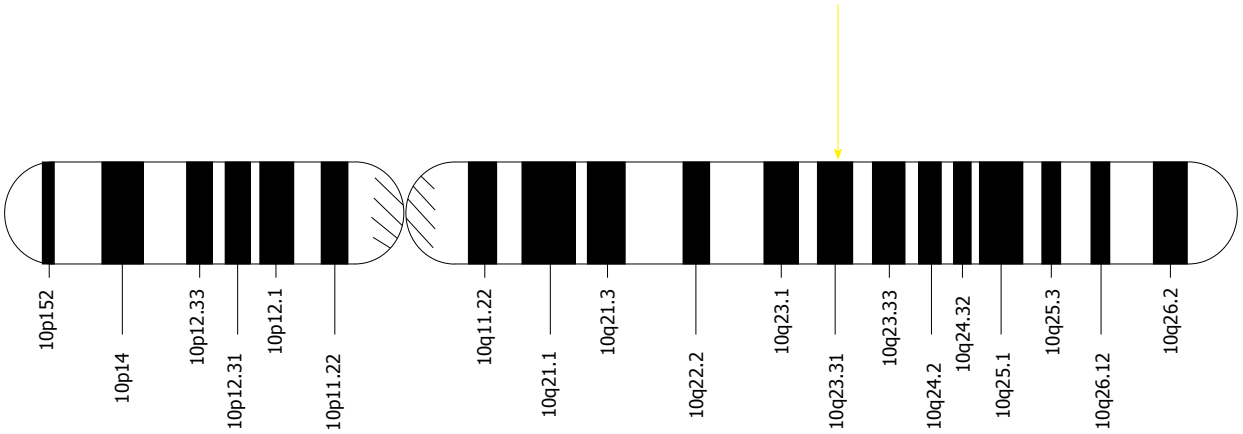

Figure 2 *PTEN* locus on chromosome 10. *PTEN*: Phosphatase and tensin homolog.

of the study was to update the understanding of the significance of this molecular marker in the current TMZ treatment era. In this study, the authors retrospectively looked at the presence or absence of *PTEN* in 155 tissue samples from patients who underwent craniotomy for resection of GBM between 2007 and 2010. The majority of these patients (80.7%) were treated with standard adjuvant radiation and TMZ chemotherapy after surgical tumor resection. What they found was that the loss of PTEN *via* LOH, mutation, or deletion was not associated independently with poorer prognosis as had been previously assumed. What they did find was that in their multivariate analysis, certain features assessed were significant predictors of worse prognosis; these included: older age ($\geqslant$ 65), poorer functional level based on KPS score, partial resection of tumor, and not instituting standard adjuvant therapy. Interestingly, the authors also found evidence that appeared to corroborate the supposition that GBM cells that had PTEN loss were more susceptible to TMZ treatment. This was suggested in an *in vitro* study that found that glioma cell lines lacking PTEN were more sensitive to TMZ treatment than PTEN possessing glioma cell lines. The thought process is that the lack of PTEN makes those cells less capable of repairing double-stranded DNA breaks effected by TMZ, and thus makes TMZ more chemotherapeutically effectual. What this implies is that the reason the authors found no statistically significant difference in patient outcome based upon PTEN loss or presence alone is because the increased effectiveness of TMZ in PTEN loss would effectively even out the outcomes between the two groups. This would appear to explain the difference found between this study and prior studies that did not evaluate patients who had undergone adjuvant TMZ treatment. Thusly, it is not entirely clear that *PTEN* loss is not an independently poor prognostic factor. Moreover, this seems to suggest that *PTEN* loss would predict a more favorable response to TMZ though the outcome would appear not to be substantively different from patients with the presence of *PTEN*.

LOH has been found to occur in increased incidence in other genes located on chromosome 10 in GBM patients. Summarily, these, alongside LOH of *PTEN* on chromosome 10q23, indicate that this phenomenon alongside mutations on chromosome 10 may possess prognostic value when found in patients with newly-diagnosed GBM. However, as discussed above, there remains more to be elucidated in the context of the contemporary treatment paradigm before such mutations may be reliably used for such prognostication purposes.

1p/19q codeletion

The next molecular phenomenon in GBM pathogenesis to be discussed is the 1p/19q codeletion. This results from an unbalanced translocation between chromosomes 1p and 19q and leads to LOH. This molecular signature has been found to have tremendous significance and clinical utility in the evolving paradigm of molecular based prognostication and treatment of high grade glioma.

There have been three randomized clinical trials that have investigated the 1p/19q codeletion in GBM and found that it actually confers a survival benefit to patients whose tumors possess this codeletion and are receiving RT and/or alkylating chemotherapy. These trials will be discussed in turn.

The first trial to be considered here is the RTOG 9402 phase Ⅲ randomized study, which included 289 patients with grade Ⅲ anaplastic oligodendroglioma or anaplastic oligoastrocytoma treated with either adjuvant RT alone or four cycles of chemotherapy (Procarbazine/CCNU/Vincristine) followed by RT (PCV - > RT)[23]. The primary endpoints were assessing differences in PFS and OS between the two arms in the study. In this, they found at 3 years out that there was a benefit in PFS in the PCV - > RT arm (2.6 years) over the RT alone arm (1.7 years); however, there was no significant difference at that time in OS (4.7 years in the RT arm *vs* 4.9 years in the PCV - > RT arm). The researchers also assayed tissue samples for the 1p/19q codeletion and assessed whether this had any bearing upon either PFS or OS. Of 201 patients assayed with fluorescence *in situ* hybridization (FISH), 93 (46%) were positive for

the codeletion. It was found that these patients had a survival benefit conferred by the codeletion over the wild type tumors. Irrespective of the treatment arm, those patients possessing the 1p/19q codeletion had a median OS of > 7 years whereas the median OS in patients without the codeletion was 2.8 years. Though treatment at this juncture did not appear to have any bearing on survival, extended follow up 2012 confirmed the better prognosis for the 1p/19q codeletion group and that PCV - > RT also appeared to improve survival over RT alone in those with the codeletion. The median OS in non-codeleted tumors was 2.6 years and 2.7 years in the PCV - > RT and RT alone group, respectively. However, patients with codeleted tumors had a median OS of 14.7 and 7.3 years in the PCV - > RT and RT alone groups respectively. Thusly, it appeared from this trial that the 1p/19q codeletion possessed both prognostic and predictive value.

Soon thereafter, the EORTC 26951 trial was conducted, compared RT alone to RT followed by six cycles of PCV (RT - > PCV) in 368 patients with anaplastic oligodendroglioma or anaplastic oligoastryocytoma randomized between the two arms[24]. Summarily, the outcome was analogous to the aforementioned trial on the primary endpoints of PFS and OS; PCV and sequential RT did increase PFS from 13.2 mo to 23 mo but had no bearing on OS (40.3 mo for PCV - > RT *vs* 30.6 mo for RT alone) at 60 mo out. The researchers in this study also used FISH to assay tissue for the 1p/19q codeletion; 78 patients (21%) were positive for the codeletion. As with the RTOG study, 1p/19q codeletion was prognostic and conferred a better outcome irrespective of therapeutic intervention. At 60 mo out, those patients possessing the 1p/19q codeletion did not reach a discrete median OS whereas those without the codeletion and treated with RT followed by PCV had a median OS of 25.2 and 21.4 mo for those treated with RT alone. Results after extended follow up of 12 years in 2012, again mirrored those of the RTOG trial at extended follow up, with RT - > PCV yielding a greater OS (no median OS reached in these patients) over RT alone (median OS of 9.3 years) in patients with the 1p/19q codeletion. This survival benefit was not seen in those patients without the codeletion; in this contingent, those receiving RT - > PCV had an OS of 25 mo and those receiving RT alone 21 mo.

A third trial known as NOA-04 conducted by the German Neuro-Oncology Group prospectively evaluated 318 patients with anaplastic astrocytoma, anaplastic oligodendroglioma, and mixed anaplastic oligoastrocytoma treated with RT, PCV, or TMZ-by the ratio of 2:1:1, respectively[25]. Patients who experienced excessive toxicity or progression after RT were then randomized to receive either PCV or TMZ, or patients with similar outcomes during or after primary treatment with chemotherapy were then administered RT. The primary endpoint was treatment failure, and 43% reached that endpoint at 54 mo out. On PFS and OS, the three groups were found to have similar results. In this study, FISH assays of tissue found 74 patients (23%) to possess the 1p/19q codeletion. When assessing these patients against wild type patients on the primary endpoint, there was, regardless of therapeutic intervention, an improvement of almost 50% in treatment failure. What should be understood, however, is that the benefits conferred by the codeletion had no bearing upon those patients with anaplastic astrocytoma. The aggregate of the studies described above show that the 1p/19q codeletion has both prognostic and predictive utility in malignant gliomas and may thereby represent a tool to guide clinicians in prognostication and treatment planning for patients with these malignancies.

IDH1/IDH2 mutations

The genes IDH1 and IDH2 are molecular markers that demonstrate prognostic value in patients with glioblastomas as well as lower grade gliomas. Isocitrate dehydrogenase (encoded by IDH1 in the cytoplasm and by IDH2 in the mitochondria) in its wild type form produces alpha-ketoglutarate[26]. Mutations in these genes encode an aberrant enzyme that turns alpha-ketoglutarate into an onco-metabolite, D-2 hydroxyglutarate. D-2 hydroxyglutarate controls the oncogenicity of IDH mutations. Based upon mutation status, gliomas may be classified as IDH-wild-type or IDH-mutant. IDH-wild-type gliomas include grade Ⅰ pilocytic astrocytomas and primary GBMs. Tumorigenesis in this case is, therefore, independent of the IDH status and is mediated by other oncogenes. IDH-mutant gliomas include grade ⅱ and grade Ⅲ gliomas as well as some secondary GBMs. What is interesting is that within a given histological class, IDH mutants carry a better prognosis than IDH wild types. For example, in WHO class Ⅳ tumors, secondary GBMs (IDH mutants) carry a better prognosis than primary GBMs (IDH wild types). An analysis of 382 high grade gliomas in 2010 found that IDH status has greater prognostic value than histological grade[27]. Thusly, it is now being realized that grouping gliomas by IDH status is more useful for prognostication than grouping by histological grade and morphology.

p53 mutations

It is well known, that protein p53, with a gene located on the short arm of chromosome 17 (17p13.1), is one of the main tumor suppressors. It is a transcription factor that activates the expression of genes that will induce the G1 cell cycle arrest in response to cell stress and DNA damage. Hence, the somatic and the germline mutations of p53 are associated with a variety of human cancers[28,29].

The Cancer Genome Atlas Network (TCGA) reported p53 mutations in 37.5% of the newly diagnosed, and in 58% of the previously treated GBM samples[30]. As far as the pathogenesis of malignant gliomas is concerned, the mutations in p53 and its regulatory pathways primarily play a role in development of secondary gliomas as

opposed to the primary glioblastomas[31-33].

Other than the cell cycle progression, several cellular processes are thought to be affected by p53 such as: the response to DNA damage, apoptosis, and the cellular differentiation and neovascularization[34]. Based upon cellular homeostasis, the p53 gene product is typically low in normal cells and increased in cells affected by DNA damage, where it exhibits a relatively short half life, being degraded by the Murine Double Minute 2 (MDM2) protein in the cytoplasmic milieu[35].

Apart from the events that mutate the p53 itself, the mutation of genes encoding its functional regulation are found in approximately 70% of GBM samples, mainly ARF, 55%, MDM2, 11%, and MDM4, 4%[36]. The MDM2 and MDM4 proteins that function as E-3 ubiquitin ligase, degrade the p53 and repress its function. It has been confirmed, that the amplification of MDM2/MDM4 proteins inactivates the transcriptional activity of p53, resulting in abrogation of its antiproliferative and apoptotic effects[37,38].

The CDKN2A locus has been shown to present another frequent mutation in glioblastomas. In addition to encoding the p16INK4a, that is a specific inhibitor of CDK4/6, this locus encodes a second protein, the p14ARF, whose expression also induces a cell cycle arrest. The p14ARF acts by binding MDM2, thus promoting its rapid destruction, and leading to the stabilization and accumulation of p53. For INK4a/ARF locus mutation, the protective, antitumorogenic role of the p14ARF is lost, due to the suppression of p53[39]. The importance of this locus is also confirmed by observation, that in mice models the homozygous deletions of both p16 and p14, are correlated with the increased progression from lower to higher grade gliomas and poorer survival rate in patients older than 50 years[40].

Molecular profiles in GBM

In 2010, the group of Verhaak *et al*[41] published results of a study in which they utilized the genomic sequences of 91 GBM patient made available by TCGA Research Center to look at patterns of gene mutations and expression in different tumors that may allow for categorization of these tumors into distinct subclasses. They found they were able to find distinct genomic patterns that hewed to a classification system that would allow them to classify any given tumor into one of four subtypes: Proneural (PNL), Neural, Classical (CL) and Mesenchymal. In addition to allowing for the distinct biology for tumors of each of these classes to be contrasted with the others, it was posited that this may have utility for prognostication and/or predicting response to treatment.

As discussed, GBM by histology is characterized as one entity. This has been found to be a considerable oversimplification that does not account for the differences in biology between different GBM tumors. Genetics have revealed that there exist multiple subtypes of GBM.

What is clinically significant here is that the biology of each subtype confers upon it differences in prognosis and/or response to treatment from the other subtypes. As to the final consensus on how many genetics-based subtypes there actually are, this remains to be determined and studies are ongoing to this end.

One such study that provided a compelling insight into what is likely to be representative of the future of GBM classification came from a group out of Belgium in 2012[42]. The authors predicated their investigation upon the results of Verhaak *et al*[41] cited above, namely exploiting unique patterns of genetic mutations to classify GBM into distinct biological subtypes that each have unique clinical characteristics. The goal of the study was broadly twofold: to devise a relatively simple assay of mutations in tumor samples for classification into one of two subgroups; and to try to ascertain biological features of tumors from each subgroup that have demonstrable value in making clinical inferences. To this end, the authors did a retrospective analysis of 100 patients with new, treatment naive GBM. They utilized immunohistochemistry (IHC) to quantitatively assay tumor samples from these patients for the presence or absence of mutations in 3 well-characterized genes in GBM-EGFR, PDGFRA, and p53. Based upon the pattern of presence or absence of mutations in these genes, the investigators were able to discern two subtypes of GBM: the CL subtype and the PNL subtype. To be sure, these subtypes had been initially described by Verhaak *et al*[38] but the association with the IHC mutational analysis done here was entirely new. The CL subtype is characterized by positive immunostaining for EGFR and is negative for p53 and PDGFRA mutations. The PNL subtype, on the other hand, is EGFR negative and demonstrates positive immunostaining for p53 and/or PDGFRA. Of the initial cohort of 100 GBM specimens, 93 were able to be quantitatively assessed for these genetic signatures. Based upon the criteria set outlined, 35 specimens were found to belong to the CL subtype and 56 were found to belong to the PNL subtype; the other 2 specimens did not stain for any of the three markers. The endpoints assessed for the patients in this retrospective analysis were PFS and OS. The former was defined as the time elapsed from the date on which the tumor was resected to the date on which the tumor was found to have recurred or if the patient died from recurrence of tumor. The latter was defined as the time elapsed from the date on which the tumor was resected to the date the patient died due to tumor progression. Summarily, the study found the following of notable clinical significance. Firstly, patients with tumors of the PNL subtype had a statistically significantly higher median OS of 10.5 mo than the median OS of 5 mo for patients of the CL subtype (P = 0.047). Similarly, a mortality risk reduction of 52% was linked to the PNL subtype when compared to the CL subtype. Hence, it was suggested that the delineation of a given GBM patient to one of the two subtypes would possess value in prognostication. Furthermore and not insignificantly, the authors demonstrated that the information needed

to make this categorization, *i.e.*, PDGFRA, EGFR, and p53 status, is relatively easily obtainable through IHC staining. Secondly, the authors found that these two subtypes possess biological characteristics conferred by their respective genetic signatures that make their response to adjuvant adjuvant chemotherapy different from one another.

Specifically, they found that temozolomide chemotherapy with radiotherapy did dramatically improve survival of patients of the CL subtype. This was not the case in that contingent of CL patients receiving radiotherapy alone, who showed no significant improvement in OS compared to patients receiving no treatment or palliative management. Interestingly, treatment modalities had quite the opposite effect on patients of the PNL subytype. These patients who received radiotherapy alone saw a significant improvement in OS over those who received no treatment or palliative management. However, the addition of temozolomide to radiotherapy did not improve survival in this subset of patients as it did in the CL patients.

Autophagy

When considering the multimodal actions of TMZ as a chemotherapy agent and contextualizing this in the problem of chemotherapy resistance, a topic of recent research interest is autophagy.

Autophagy, known as type Ⅱ programmed cell death, is a catabolic process during which cells self-digest intracellular organelles. When allowed to go to completion, autophagy results in cell death[43,44]. Biologically, it serves two functions: as an intracellular mechanism of disposing of damaged organelles and proteins, and for catabolism of substrates during cellular stress in order to generate energy needed for cell survival. As may be intuited, persistent autophagy does in many cases result in cancer cell death. However, there is also mounting evidence that autophagy may also drive the damage response that cancer cells use to avoid death when exposed to metabolic and therapeutic stresses.

Knizhnik *et al*[45] demonstrated in glioma cells that TMZ can induce cell death *via* a complex process between apoptosis, autophagy, and senescence. Senescence represents a state when viable cells stop synthesizing DNA with the unknown endpoint of either survival or death. They demonstrated that TMZ - induced cell death could be accomplished by two mutually exclusive pathways: by apoptosis alone (*via* the caspase-mediated pathway) or by autophagy followed by cellular senescence. It was found that the autophagy pathway inhibited the appositional apoptosis pathway, and the cells progressed to senescence. Thus, it is proposed that autophagy may be a survival mechanism whereby gliomas undergo senescence rather than immediate death *via* apoptosis when therapeutic doses of TMZ are used. Knizhnik *et al*[45] also found that autophagy, senescence, and apoptosis of glioma cells occurred at 72, 96, and 120 h after TMZ exposure, respectively.

As a result of TMZ-induced autophagy, it is possible that the high recurrence rate in glioblastoma patients and the unsatisfactory clinical survival rate might not only be due to the resistant mechanisms of tumors such as MGMT and deficiency of MMR but also due to autophagy allowing the tumor to survive where it should otherwise undergo apoptosis. As such, investigations are underway to see if adjunctive treatment with an autophagy inhibitor may enhance the beneficial therapeutic effects of TMZ for patients with GBM.

Vascular endothelial growth factor

GBM is one of the most vascularized human tumors and, alongside high expression of various proangiogenic factors, vascular proliferation is one of its defining pathologic features[2]. GBM cells produce proangiogenic factors; one of, if not the, best characterized of these is vascular endothelial growth factor (VEGF).

VEGF consists of a family of 5 glycoproteins: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor. These factors bind with their corresponding tyrosine kinase receptor (VEGFR-1, VEGFR-2, and VEGFR-3) and activate a signal transduction cascade that results in the development of angiogenesis, increased vascular permeability, and lymphangiogenesis. Of these, VEGF-A plays the greatest role in tumor angiogenesis along with tumor cell proliferation and migration. Thusly, elevated levels of VEGF-A in patients with cancer--specifically that of breast, lung, colon, uterus, and ovary--confers a graver prognosis[46].

Bevacizumab is a humanized monoclonal antibody to VEGF-A[47]. This antibody prevents the interaction of VEGF with target receptors VEGFR-1 and VEFGR-2 on the surface of endothelial cells. This in turn prevents downstream signaling that would normally induce tyrosine phosphorylation and the subsequent cascade of signal transduction events that would lead to endothelial cell survival, proliferation and vascular permeability. The composite effect of causing regression of existing microvasculature, inhibition of new vessel growth and normalization of the surviving vasculature (which leads to reduced vascular permeability and reverses peritumoral edema) bears a particularly germane pertinence to GBM. In the United States, bevacizumab has been approved for recurrent GBM based on studies that showed improvement in PFS but not OS[47].

In February 2014, Chinot *et al*[48] published randomized, double-blinded, placebo controlled trial on newly diagnosed GBM patients where they compared standard radiotherapy and TMZ for newly diagnosed GBM with or without bevacizumab. The study met the first primary endpoint of improved median PFS with statistical significance ($P < 0.0001$), finding a 4.4 mo improvement in median PFS of the experimental group (10.6 mo) over the control group (6.2 mo).

The OS at 1 year ($P = 0.049$) was 72.4% and 66.3% in the experimental and control groups, respectively. At 2 years ($P = 0.24$), the OS was found to be 33.9% and 30.1%, respectively, which was

not statistically significant. The experimental group receiving Bevacuzimab maintained a longer quality of life and performance status and required less steroids. However, the study noted that there was a clearly greater number of clinically significant adverse events in the Bevacizumab group than the control group.

The RTOG 0825 study, published in the same month as the study by Olivier *et al*[49], came to a similar conclusion: namely that adding bevacizumab to standard of care RT/TMZ provided discernible benefits for PFS but not for OS. Notable adverse effects in the bevacizumab group were hypertension, thromboembolic events and intestinal perforation, consistent with previously reported side effects of this medication.

The BELOB trial by Taal *et al*[50] out of Europe investigated three lines of therapy for patients with recurrent GBM: single-agent bevacizumab, single-agent lomustine and combination therapy with bevacuzimab plus lomustine. Results demonstrated 9-mo OS to be 43% in the lomustine group, 38% in the bevacizumab group and 59% in the combination group. Extrapolating from these results, the authors strongly questioned the role for single agent bevacizumab in recurrent glioblastoma. However, it provided a compelling indication for further investigations of combination bevacuzimab with lomustine, particularly in a phase Ⅲ trial.

The aggregate of data from clinical trials on bevacuzimab for newly-diagnosed and recurrent GBM reveals that the proposed mechanism of action of bevacuzimab in antagonizing the VEGF pathway is not enough on its own to explain the observed results. It has led investigators and the scientific community to realize that there are much more complex regulatory mechanisms in angiogenesis at work than previously recognized.

Hypoxia and treatment resistance

Recent evidence has indicated that prolonged anti-angiogenic treatment leads to development of progressive hypoxia in tumor tissues which in turn has led to the recognition of an entirely novel paradigm of treatment resistance. VEGF blockade of its own causes only a small reduction in tumor burden but does induce a strong depletion of large and intermediate-sized blood vessels with a subsequent reduction in vascular leakage and intratumoral blood flow.

This result in a hypoxic microenvironment within the tumor which is proposed to provoke significant tumor cell invasion.

Hypoxia-inducible factor-1 (HIF-1) is a transcriptional complex belonging to a family of transcriptional factors known as hypoxia inducible factors (HIFs) that is activated in response to hypoxia and growth factors. HIFs are heterodimers composed of an oxygen-sensitive HIF-alpha subunit and a HIF-beta subunit. Under normal homeostatic cellular conditions, HIF-alpha binds to the tumor suppressor protein von Hipel-Landau (vHL), which leads to degradation of HIF-alpha.

However, under conditions of hypoxia, there is an abrogation of the interaction between HIF-alpha and vHL as a result of which HIF-alpha gets stabilized. This leads to dimerization of HIF-alpha which then allows it to bind to hypoxia responsive elements on promoters of genes inolved in promoting cell survival, motility and metabolism. The activation of HIFα also plays a regulatory role in the expression of VEGF and inducible nitric oxide synthetase facilitating angiogenesis and the tumors cell's access to the circulatory system. Two HIFα subunits, HIF-1α and HIF-2α are primarily responsible for regulating tumors adaptation to hypoxia. HIF-1α is widely expressed in several tissues, while HIF-2α has a more restricted expression pattern and is associated with cancer initiation or tumor progression. Thusly, HIF-1 plays a central role in tumor progression, invasion, and metastasis. Indeed, overexpression of the HIF-1α subunit has been observed in many human cancers and is associated with a poor prognostic outcome with conventional treatments[51].

Preclinical trials of recent have revealed some very intriguing characteristics of tumor vasculature. Three major mechanisms have been proposed for the development of new tumor vasculature: proliferation from preexisting vessels, colonization by circulating endothelial cells or colonization by proangiogenic bone marrow cells. This last phenomenon has come to be denoted as vasculogenesis[52].

In specific, vasculogenesis itself depend on three major pathways: (1) mobilization and recruitment of proangiogenic bone marrow derived cells (BMDCs) into tumor milieu; (2) retention of these BMDCs in hypoxic tumor tissues; and (3) vascularization dependent on $CD11b^+$ myelomonocytic cells.

Hypoxia leads to induction of the transcription factor HIF-1 which has been shown to be a major recruiter of BMDCs to tumors including GBM. Retention of these cells is dependent on secretion of stromal cell derived factor-1 (SDF-1, CXCL12) which binds its receptor, CXCR4, on the BMDCs. Thus has been elucidated the link between hypoxia in GBM and vasculogenesis.

This in turn has led researchers to propose the means by which bevacuzimab engenders treatment resistance.

The proposed hypoxia model as discussed has been further supported by studies looking into inhibitors of the modulators of vasculogenesis. In xenograft models, the HIF-1 inhibitor NSC-134754 and AMD3100, an inhibitor of the SDF-1/CXCR4 interaction, compellingly found little to absent tumor regrowth following irradiation[53].

Hypoxia has been also been proposed as a means of activating autophagy, the lysosomal degradation pathway that, as discussed earlier, likely promotes tumor cell survival[54]. The mechanisms by which hypoxia induces autophagy need elucidation, but the finding that BNIP3, a downstream target of HIF-1α, is essential to hypoxia-induced autophagy suggests a likely mechanism.

Cancer stem cells

An additional important developing point of interest with therapeutic potential is the identification of cancer cells with stem cell-like properties. It has been hypothesized that a subset of cells known as the cancer stem cells exist within a tumor with stem cell like properties and can initiate primary tumors as well as recurrences by way of their self-renewal capacity and inherent resistance to therapeutics.

Glioblastoma contains multipotent tumor stem cells (GSCs) that could be responsible for populating and repopulating tumors.

Specific criteria are required to define GSCs: (1) the ability to self renew; (2) the ability to differentiate into different lineages (multipotency); and (3) the ability to initiate tumors in animal models which recapitulate the original disease phenotype and heterogenicity[55,56].

Multipotent neural stem cells have the ability to differentiate into neurons and glia (astrocytes and oligodendrocytes). Physiologically, stem cells have a long life expectancy and divide frequently which makes them more susceptible for tumorigenesis.

The process of neurogenesis occurs in two major regions of the adult brain: the subventricular zone of the lateral ventricles (SVZ) and the subgranular layer of the hippocampal dentate gyrus[57].

Neuronal stem cells (NSCs) are regulated by the orchestration of intrinsic factors with extrinsic signals from surrounding microenvironment, defined as the neurogenic niche. A niche represents a specialized anatomic compartment formed by cellular and acellular components that integrates local and systemic factors, supports maintenance and survival and actively regulates the function and proliferation of NSCs.

It has been hypothesized that once neurogenic niches house NSCs (which have a relatively large chance of becoming cancerous cells) and support maintenance, survival and proliferation, they become vulnerable sites for growth and proliferation of transformed cells. It is believed that the SVZ gives rise to the highest number of glioblastomas and this has led to efforts looking at this cell population as a potential therapeutic target.

The sole process of neurogenesis depends on a complex cascade of molecular signaling pathways. These candidate pathways include Notch[58], bone morphogenic protein[59], Wnt[60] and sonic hedgehog (Shh)[61].

Blockage of Notch signaling with γ-secretase inhibition, inhibits self-renewal, and causes CD133+ cell depletion[62]. Transforming growth factor-β (TGF-β) signaling promotes GSC self-renewal[63]. Shh signaling (important during embryonic development) plays an important role in GSC maintenance by promoting self-renewal and expression of stem cell genes[64], whereas blockage leads to apoptosis, delay in tumorigenesis and inhibition of GSC self-renewal and migration[65].

Similarly to that of normal stem cells, GSCs are found in a microenvironment that provides ideal condition for tumor maintenance. The tumor perivascular niche is composed of heterogeneous cell groups, including astrocytes, endothelial cells, macrophages, microglia, non-tumor initiating cells, and, indeed, tumor stem-like cells[66].

GSC chemotherapy resistance and radiotherapy resistance

Multiple mechanisms leading GSCs to chemo-resistance have been identified in pre-clinical studies. These include: increased activity of ABC-type transporters present on the cell surface that extrude chemotherapeutic agents to the extracellular space[67]. These chemo-resistant cells have been identified in GBM cells *via* flow cytometry with a specific pattern of expression of surface antigens (CD133+, CD117+, CD90+, CD71+, CD45+)[68]. Further corroborating the important role GSCs have in chemo-resistance, CD133 is highly expressed in recurrent tumors and transcriptional analysis of these cells demonstrates concurrent over-expression of anti-apoptotic genes[69]. Parada *et al*[70] applied these findings and showed that a restricted Nestin+ GSC population could regenerate tumors after being treated with temozolomide. Others have attempted selective ablation of this cell population and this only led to tumor growth arrest, supporting the hypothesis that GSCs resist current standard chemotherapy and have intrinsic properties of chemo-resistance. In addition to the above, GSCs have slow cell cycles, generally quiescent and are immune to exposure to chemotherapy because these traditionally target actively cycling cells. GSCs also have the ability to evade irradiation with the development of clones that over-express GSC markers as well as triggering over-activation of the Notch and TGF-β signaling pathways[71,72].

CONCLUSION

GBM has historically been and indeed remains a formidable challenge for clinicians and has maintained a grim prognosis not much changed from the very inception of conventional treatment. This is despite a profusion of significant recent discoveries regarding its unique biology and intricate molecular pathogenesis. However, with the elucidation of these recent and ongoing findings, there are a number of exciting studies underway investigating entirely novel treatment modalities that exploit these recent revelations. It is expected that with fruition of validated results in animal models and progression to phase Ⅲ clinical trials, a veritable revolution will take place in both the diagnosis and treatment of this most malignant of primary brain cancers.

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World Journal of Neurology (*World J Neurol*, *WJN*, online ISSN 2218-6212, DOI: 10.5316) is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

WJN covers topics concerning neuro-oncology, electroneurophysiology, cerebrovascular diseases, epilepsy, cognitive impairment, myopathy and peripheral neuropathy, degenerative diseases, infectious diseases, demyelinating diseases, immunological diseases, genetic/metabolic diseases, affective disorders, headaches, sleep disorders, interventional neuroradiology, minimally invasive therapy, rehabilitation, diagnostic imaging, evidence-based medicine, epidemiology and nursing. Priority publication will be given to articles concerning diagnosis and treatment of neurological diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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EDITORIAL OFFICE
Jin-Lei Wang, Director
Xiu-Xia Song, Vice Director
World Journal of Neurology
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: editorialoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
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EDITORIAL

Challenge of the translational neuroscience

Rodrigo Ramos-Zúñiga

Rodrigo Ramos-Zúñiga, Translational Neurosciences Institute, Department of Neurosciences, CUCS, Universidad de Guadalajara, Guadalajara, Jalisco 44340, México

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Correspondence to: Rodrigo Ramos-Zúñiga, MD, PhD, Translational Neurosciences Institute, Department of Neurosciences, CUCS, Universidad de Guadalajara, Guadalajara, Jalisco 44340, México. rodrigor@cencar.udg.mx
Telephone: +52-33-10585271
Fax: +52- 33-36402277

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Abstract

The development of Neurosciences in the last few years has changed a set of paradigms in the production of knowledge, from which new scenarios have arisen in the understanding of the structure and function of the human nervous system, as well as in some of the most relevant diseases involved. Nonetheless, the impact of all the scientific information on this topic has played a limited role in the proposals in the diagnostic, therapeutic, rehabilitation and social reintegration fields, when the effect on the daily life of patients that have a neurological impairment is considered. Thus, the emergence of translational science is an alternative for a more direct and pragmatic link that allows the connection between basic research and applied research, and in the short term will achieve results that can be promoted in the communities. In addition, this process involves an interaction with technological development and transfer following a global knowledge management model. Every discipline in the neurological sciences field poses different critical challenges to tend to the new epidemiologic profiles. emerging in areas such as neurodevelopment disturbances found in the pediatric population, trauma and addictions in the young, as well as neurodegenerative diseases in older adults. This model reviews the demands from society, expecting more compelling results from the scientific community, particularly in creating strategies that actually change the natural course of neurologic diseases from the bench to the bedside.

Key words: Medical research; Neurology; Neurosurgery; Neuroscience education; Neuroethics; Translational medicine

Core tip: The society of knowledge has expanded with information produced all over the world. But unfortunately, only a small part of such knowledge has had an impact on decision-making pertaining health, and on the ability to solve specific problems in a given population. Translational Neurosciences represent an innovative proposal for a direct line between basic research, applied research, technology transfer and knowledge management for the resolution of a specific problem in the neurological sciences, either in diagnosis, therapy, rehabilitation or social integration. This design requires commitment with education and training of human resources in Neurosciences from a proactive and innovative viewpoint.

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INTRODUCTION

The control of certain emerging diseases and proposals for prevention has been fostered by the growth of science and technology. New innovative strategies have been devised that have favorably changed the worldwide standards of life quality and life expectancy.

However, the creation of new information does not necessarily mean that scientific knowledge is duly applied at every public health level. The society of knowledge has expanded with information produced all over the world, but unfortunately, only a small part of such knowledge has an impact on decision-making pertaining health, and on the ability to solve specific problems in a given population.

The gap between the production of knowledge and its application in daily life and health issues in the communities is today a major challenge[1,2].

TRANSLATIONAL MEDICINE

One of the best methodological strategies to rethink the paradigm of the production and application of knowledge is found on translational science. It comes forth under the promise of change and a direct short-term impact on the interaction between knowledge and problem resolution. For pragmatic purposes in health sciences this precept is applicable to the concept of Translational Medicine[3].

A crucial fact from its origin is that it arises from the same demand of the so called translational epidemiology, which has been profiled as an emerging condition that supports. That is due to the pertinence of its origin, to try get the different fields of biomedical research to have an impact on public health at the shortest possible term[4].

This protocol makes it more feasible to allow for the transition of basic research into action and resources that are specifically an answer to clinical needs in a more timely and specific way.

One of the most accepted definitions states that it is a process by which the scientific research discoveries are transformed by clinical projects that allow for new treatments promote diagnosis, treatment and disease prevention in the short term. In the scientific literature it is identified as a collection of monographs that are described in a format called "from the bench to the bedside".

This proposal is put forth as bidirectional, since multidisciplinary work is a commitment from the members of the basic research team and the clinical investigators, and it also allows to rethink the prevailing problems in clinical work that need support from basic science. In other words, the proposal strengthens competences and promotes resolution of concrete problems punctually and specifically, under a strategy of multidisciplinary leadership and teamwork.

The way of thinking is linear, dynamic, bidirectional, with innovation in the pivotal points and the traditional scope of biomedical research, where the commitment lies on the idea that knowledge coming from a reality must go back to it and transform it, beyond a mere description under the perspective of scientific rhetoric[3,4].

Its field of action is broad, since on one hand it requires, due to its own nature, an operative link with technology transfer, creation of patents, promotion of intellectual property and even entrepreneurship. On the other hand, it requires an accurate link with knowledge management and the impact on management guidelines and decision-making in a specific problem in the realm of public health.

TRANSLATIONAL PERSPECTIVE IN NEUROSCIENCE

Basic neurosciences have had an extraordinary progress in the last 30 years which has modified the frontiers of knowledge and has changed paradigms in neurogenesis, molecular structure, genetics, network functions and specific circuits. This has led to better understanding of basic science and neural pathophysiology and neuropathology, having a high impact on clinical neurology and cognitive neurosciences. In spite of all that, the molecular mechanisms underlying the different diseases and their respective treatment have not been totally elucidated, therefore, many questions remain unanswered in every branch of the knowledge[5].

In view of these events, societies and some political leaders have questioned the fact that although brain research has been consistently supported, compelling advances have not been obtained in the treatment or prevention of the most disabling cerebral diseases in human beings[6,7].

Aside from the complexity in the analysis of the human brain, transfer of neuroscientific knowledge to the biosanitary field has not been easy. This is related in terms not only of primary research and its application, but also including some other factors such as the so called T2 translational research that puts forth limitations and barriers so that the relevant products of basic and clinical research can move on to be applied in health systems at short term, due to feasibility and cost-benefit implementation difficulties[8].

This feature has a direct impact on the creation of new treatment strategies for neurologic diseases of every sort, which are the ones that account for a high rate of disability. Also have an effect on people's independence and the consequent social and economic impact on public health systems.

This scenario in Neurosciences is an overt example where we find an imperative need to transfer knowledge to the bio-health sector, which has led the Health

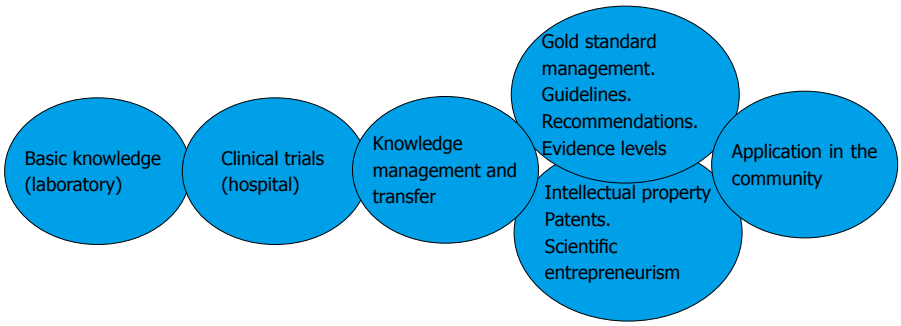

Figure 1 Lineal representation that links the different phases in the production and application of knowledge from the translational perspective.

Institutes - National Institutes of Health, INH- to redirect the funds from research lines to advanced projects in the field of Translational research, in view of a predictable social and public health impact[9].

Consequently, the scientific world has undertaken a series of steps for the creation of a more integrative multidisciplinary strategy between Basic and Clinical Neuroscience.

Ever since the decade of the brain[10], and up to the implementation of more recent study projects such as the BRAIN project[11], and the recently created project of the human connectome[12], every effort in the different scientific communities has the goal of conducting research to discern the map of the human brain and its implications for disease in different clinical areas, such as Neurology, Neurosurgery, Neuropsychopathology and Psychiatry. However, the real challenge must be to discuss the goals with the greatest reach that will be able to link the different research levels with concrete applications at short term, that can actually have direct impact on health and that also promote public healthcare and prevention policies[13].

In the case of Neurosciences, the demand is for almost immediate application. First, due to the large amount of scientific information that is produced in the field. Second, because of the still perceptible distance between research and its effects, since it is considered that such impact has not been big enough to change the epidemiology of diseases that involve the human nervous system and that are a challenge and an emergency in worldwide public health[14].

Translational Neurosciences represent a new proposal for a direct line between basic research, applied research, technology transfer and knowledge management for the resolution of a specific problem in the area of neurological sciences, either in diagnosis, therapy, rehabilitation or social integration (Figure 1).

This feature gives a reference framework according to world trends, but also sets the limits and scope concerning specific topics in basic science and clinical work. Also, this design requires commitment for education and training of human resources in this topic from a proactive and innovative viewpoint. The model pursues not only to be involved in the transformative practice offered by translational science, but also to promote proactive mechanisms of knowledge management that can support the algorithms in the decision making. Also, other process around health and public policies at a global level with more compelling scientific strength, but supported on local needs.

TRANSLATIONAL IMPACT IN TIME

Some of the limitations of this process are the so called gaps or death valleys, where time sequence plays a crucial role. They involve extended laboratory research time periods due to their own nature, with the consequent loss of continuity and validity on a specific subject of knowledge to be applied in clinical settings (Long periods between biomedical research phases I-II-III, approval delays, flaws in research product feasibility and availability, questions in cost-benefit balance). Additionally, it must be considered that regulatory criteria and scientific rigor itself can occasionally reduce the implementation of new projects and their clinical application, in spite of having been successful and promising in the research laboratory.

Under emerging conditions, these time periods are shortened by common sense and the existing social pressure under certain circumstances such as epidemics. Or due to the individual decision of patients about using experimental therapies at their own risk even if they are still not approved. This is particularly obvious in case of diseases for which there are no curative treatment options, as happens in certain neurodegenerative diseases[5].

Nevertheless, this condition must not misrepresent the fundamental premise in Bioethics and now Neuroethics by assuming that the viability of the translational process to apply knowledge at short term could compromise the values, rights and responsibilities in protecting health and integrity of patients as a primary endpoint[15,16].

CHALLENGES IN NEUROSCIENCES

The World Health Organization (WHO) has proposed studies that have led to public policies on global health, based on surveys and epidemiological analysis in the area of neurological sciences. Agreements among different institutions, have integrated a proposal concerning

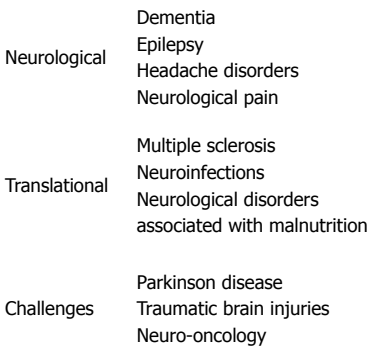

Figure 2 The most relevant challenges for global health in neurosciences for the future years.

neurological disorders as a public health challenge[1].

Under the basic public health principles and knowledge management, a very specific proposal has been postulated involving the most relevant challenges for global health, not only for today but also with future projection under different schemes for years to come: (Figure 2).

In this illustrative outline a methodological design is set forward, contemplating basic public health precepts, such as global health, community health, prevention, monitoring, epidemiologic surveillance and social conditions to attain equitable health.

According to this project, decisions for primary prevention (measures to avoid the onset of disease), secondary prevention (accurate and timely diagnosis, appropriate treatment and management of risk factors), and tertiary prevention (rehabilitation, palliative care, treatment for complications, patient and caretaker education, self-help groups, reducing stigma and discrimination and fostering social integration) are duly stipulated[1,17].

If we analyze the new challenges for Neurosciences as a projection into the future, a new position and a new strategy are required, so that in a practical way there can be a direct reflection on the epidemiological framework, under a translational format[18]. An example is the case of the comparative analysis applied to the use of a helmet and its effect on consequences of traumatic brain injury as evidence[19].

On the other hand, it is also pertinent that the proposals to solve these problems should have a long term and obvious impact on functional quality and the quality of life of subjects who have neurological disorders. Consequently, the translational challenges of the new research projects in Neurosciences must also find a way to have a favorable impact or effect on the disability and physical limitations, cognitive sequelae, behavior sequelae, communication and daily life activities as well as psychosocial issues in patients. All, within a global social context and under a scheme that is associated with the commitment of preserving health as a crucial component of the individuals universal rights.

CONCLUSION

It is crucial that under this vision of things new educational schemes that strengthen the training of human resources on basic neurosciences and all neurological sciences should be created, with a clear commitment of collaboration in "translational teams". In such a way that they are able to update the core curricula (undergraduate and postgraduate), continuing medical education, accreditation and certification; the creation of international networks, the integration of non-medical professionals, the rational use of new technology and consideration of the projective public healthcare and emerging conditions.

Translational Neuroscience must face the challenge of growing from methodological and technological innovation to the production of new knowledge through basic and applied research, promoting discipline and leadership. The exchange and optimization of research work must consider knowledge and transfer management as a priority, for the benefit of community health.

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FRONTIER

Medicine in the future - with subspecialists in medullary neurology and brain dentistry

J Howard Jaster

J Howard Jaster, Department of Medicine, London Corporation, Grand Prairie, TX 75050, United States

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Correspondence to: J Howard Jaster, DMD, MD, Department of Medicine, London Corporation, 570 Bridle Path, Suite 1117, Grand Prairie, TX 75050,
United States. harbert38104@yahoo.com
Telephone: +1-901-7347414

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Abstract

The solitary tract nucleus of the medulla with its limited watershed vascular capacity may occasionally be the focus of transient ischemia caused by the increased metabolic demands associated with frequent and intense neuronal stimulation from other organs and other parts of the brain. Case reports have suggested that these ischemic changes may sometimes result in the initiation of intense autonomic discharges, which can occasionally be fatal. Therapeutic interventions for the medulla oblongata are hampered by its limited accessibility. Systemically administered pharmaceuticals may have some usefulness in future years. Previous experience with vagus nerve stimulation in the treatment of epilepsy suggests that it may have some usefulness in stabilizing medullary autonomic discharges. Computerized electronic stimulation of other cranial nerves may be helpful as well, especially the chorda tympani nerve, and may be most easily accomplished from implanted dental appliances, especially molar modules, transmitting signals *via* secondary transmitters procedurally placed on cranial nerves. Future technology may enable wireless signaling from the implanted dental appliance to the secondary transmitter placed at the nerve site. By the year 2050 subspecialists in medullary neurology and brain dentistry may use computerized electronic stimulation of cranial nerves to prevent sudden unexpected death and treat "chest pain from the brain".

Key words: Solitary tract nucleus; Ischemic autonomic umbra; Medulla oblongata; Molar module; Chorda tympani nerve; Medullary brain lesion; Medullary neurology; Chest pain from the brain; Sudden unexpected death; Brain dentistry; Vagus nerve stimulation

Core tip: Medical investigators in the 21st century have reported numerous cases in which the presence of a small medullary brain lesion was associated with sudden unexpected death. Many such medullary lesions have otherwise produced only minor clinical symptoms and have in themselves been previously considered relatively harmless. Many victims have been considered healthy prior to sudden death, and the medullary brain lesions were incidental discoveries at autopsy, with no other causes of death identified.

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MEDULLARY NEUROLOGY

Medullary neurology, as described here, is a branch of medical science concerned with the medulla oblongata and its disorders. The term may bring to mind the work of several 19th century neurologists, such as Wallenberg, who described stroke syndromes resulting from occlusive vascular disease affecting different parts of the vertebro-basilar artery system and sometimes related to syphilis. Some of those infarctions involved autonomic fibers located in the medulla oblongata, and resulted in focal autonomic deficits involving the face. Also described in that era was medullary brain irritation resulting in fulminant systemic autonomic phenomena affecting thoracic organs. In 1861 Brown-Séquard[1] discussed his own clinical observations in the context of then recent laboratory reports by physiologists that "sudden irritation of the medulla oblongata often produces arrest of the heart's action", but that this can be blocked by sectioning of the vagus nerve.

The concept of "occlusive cerebrovascular disease as a cause of strokes" subsequently flourished in clinical practice, and the names of those 19th century investigators remain in today's medical lexicon as eponyms for specific neurological deficits. But the concept of "medullary irritation as a cause of fulminant systemic autonomic phenomena" smoldered and never caught fire. Certainly when someone drops dead unexpectedly in the year 2015, the last thing that anyone considers as a possible cause of death is a cardiac arrest resulting from medullary irritation from a small medullary brain lesion in the absence of hemorrhage or mass effect.

That may be about to change - not because medullary brain lesions, as traditionally defined, are so common, but because our traditional clinical concepts regarding ischemic lesion formation in the brain medulla fail to acknowledge its dynamic interactions with the heart, other organs, and other parts of the brain and particularly that those interactions in themselves may occasionally initiate the formation of focal ischemic medullary brain lesions by exciting some areas of the medulla beyond the fixed capacity of their watershed vasculature to supply metabolic nutrients.

SUDDEN DEATH

Medical investigators in the 21st century have reported numerous cases in which the presence of a small medullary brain lesion was associated with sudden unexpected death. Many such medullary lesions have otherwise produced only minor symptoms and have in themselves been previously considered relatively harmless. Among the most common, and pathologically benign, were those caused by small infarctions, sometimes associated with diabetes, hypertension, and sleep apnea. But a wide variety of infectious and inflammatory illnesses, as well as multiple sclerosis, have been discovered involving the brain medulla in the setting of sudden unexpected death[2]. Excluded, however, from this phenomenon were large brainstem hemorrhages, infarctions, and compressive lesions, all otherwise long known to cause sudden death.

Clinical cases of sudden unexpected death related to medullary brain lesions, specifically following cardiac arrest[3-7] or following respiratory arrest[8-17] have been reported in approximately equal numbers[2], and all age groups have been involved[2]. In many descriptions of sudden unexpected death related to medullary brain lesions the victims had been considered healthy prior to death, and the medullary brain lesions were incidental discoveries at autopsy, with no other causes of death identified. Pathological findings suggest that sudden unexpected death related to medullary brain lesions is most commonly related to lesions which include specific anatomical regions within the medulla: the solitary tract nucleus in all age groups[10,18,19], and several additional medullary areas specifically in infants[20-22]. The solitary tract nucleus is a medullary autonomic recipient of sensory afferent impulses from organs in the chest and abdomen through the vagus nerve, and it is also involved in the sensation of taste in the anterior tongue through the chorda tympani nerve. Anatomical medullary brain lesions have been thought to induce physiological autonomic abnormalities resulting in sudden death. The mechanism is unknown[8].

Pathological findings from a large number of cases taken together have led some investigators to suggest that sudden infant death syndrome may be a subset of sudden unexpected death related to medullary brain lesions[3,23-25]. This implies that biochemical medullary brain lesions may also induce physiological autonomic abnormalities resulting in sudden death, in as much as an abundance of sudden infant death syndrome research in recent years has focused on medullary neurotransmitter abnormalities, especially involving serotonin[26]. Similarly in adults, a recent neuropathology study[27] of sudden death in various subpopulations of multiple system atrophy found that depletion of medullary serotonergic neurons was associated with sudden death, the leading cause of death in multiple system atrophy. And while studying seizure disorders, a few investigators have speculated that sudden unexpected death in epilepsy may also be a subset of sudden unexpected death related to medullary brain lesions[2] based in part on the autonomic stigmata of associated seizure activity, including central apnea and laryngospasm[8,17,28].

CHEST PAIN FROM THE BRAIN

Sudden death probably occurs infrequently compared to the many non-fatal manifestations of abnormally functioning autonomic nuclei related to small medullary brain lesions. Non-fatal events may be experienced by patients as

symptoms in the chest, abdomen[7], and elsewhere; yet in these instances the primary illness is located in the medulla and not in the organ where symptoms are experienced. Symptoms may include nausea and vomiting[29], pain in the chest or epigastrium[7], and shortness of breath[13,15]. An occasional manifestation is Ondine's curse, a syndrome of sleep apnea with preserved wakeful voluntary control of respiration occurring after some medullary infarctions[16] and in association with other types of medullary lesions[12], including medullary plaques in multiple sclerosis patients who subsequently died during their sleep[9]. Most patients with medullary brain lesions will likely not experience sudden death, but the risk of sudden unexpected death in patients with medullary brain lesions is unknown.

ISCHEMIC AUTONOMIC UMBRA

The findings of case reports suggest that in some medullary autonomic nuclei, focal areas of parenchymal tissue, often dendritic, may become ischemic when excessive neuronal stimulation results in transiently elevated metabolic requirements exceeding levels that can be supported by the perfusion generated from maximum vascular flow in a fixed watershed area. Such focal medullary ischemia and infarction are often not related to vascular occlusion, or to vascular disease in general, as much as to a mis-match of accentuated metabolic activity and vascular flow which is insufficient in this somewhat unusual physiological setting. Small vessel disease, however, related to diabetes or hypertension may be a contributor. As evidence of watershed vascular insufficiency, investigators have reported small infarctions in watershed areas of the solitary tract nucleus[10,19].

Such pathological findings suggest that increased metabolic requirements and the formation of medullary ischemic lesions may be induced by repetitive neuronal stimuli originating in autonomic sites outside of the medulla, such as during intermittent episodes of heart failure[10,19]. Some focal dendritic sites on medullary autonomic nuclei capable of receiving visceral sensory stimulation *via* afferent neural pathways may experience the formation of ischemic lesions when this stimulation becomes persistent and repetitive. At the convergence of a limited vascular capacity and accentuated metabolic requirements is the spatial distribution that might be called an "ischemic autonomic umbra", the converse of an ischemic penumbra. Chronic and severe neuronal bombardment from outside the medulla may in some instances result in the formation of a small anatomical ischemic lesion or infarction, which may then itself trigger a significant systemic physiological event involving thoracic organs, possibly fatal, or possibly not[10,19].

PATIENT CARE

In today's world many patients with medullary autonomic lesions would never receive a neurological evaluation because their symptoms would not seem appropriate for referral to a neurologist. Additionally, the clinical recognition of medullary autonomic lesions often does not fit the traditional clinical neurology construct of identifying deficits in motor function, sensory function, or cognition, all of which may remain essentially normal in these patients. And medullary brain imaging is often not helpful due to bony artifacts and inadequate resolution capability.

In the year 2050 patients with chest and abdominal complaints will probably undergo evaluation specific to those areas, just as they would today. But if testing is negative, some will likely be considered candidates for medullary brain evaluation. Initial diagnostic testing of the patient possibly by magnetic resonance imaging, positron emission tomography, ultrasound, nuclear medicine, and/or other modalities available at that time will establish a profile of initial conditions within the brain medulla regarding lesions, general tissue architecture, and regional blood flow patterns. A computerized 3-dimensional reconstruction of this information will prepare the patient for treatment, which may include brain dentistry, following any acute management that might be necessary. Essential to the development of medullary neurology will be advances in non-invasive radiology that will yield 3-dimensional dynamic imaging of both medullary parenchymal tissue and medullary vasculature in significant detail, exceeding the resolution capability that is available today.

BRAIN DENTISTRY

In the context of medullary neurology, computer-assisted dental applications may become important for two reasons. First, some of the sensory input that terminates in medullary nuclei begins in the peri-dental and peri-oral anatomy, just as some peri-oral motor activity emanates from medullary motor nuclei. And second, the relative proximity of the mouth to the brain together with its accessibility makes the mouth a location in which to install implantable appliances that might potentially be used to enhance medullary blood flow, stabilize electrical activity in the medulla, and perform other functions as well. Brain dentistry, as described here, is a branch of medical science concerned with using dental applications in the treatment and monitoring of brain disorders.

ELECTRICAL STIMULATION

As a precursor of brain dentistry, the use of electrical stimulation in the management of neurological illness is still in its infancy but has attained several important milestones. Brain dentistry will likely begin by utilizing electrical stimulation of cranial nerves and building on the experience gained from the use of vagus nerve stimulation. Vagus nerve stimulation has been successfully used in the treatment of epilepsy; and in epilepsy patients it has also been shown to increase blood flow in the medulla as well as in other areas of the brain[30]. Many sensory autonomic afferent fibers of the vagus nerve begin in the chest and abdomen and terminate in the solitary tract nucleus in the medulla. Vagus nerve stimulation follows those fibers to

this critical area, and then apparently goes on to stabilize electrical activity throughout the cerebral cortex to prevent seizure activity by a mechanism yet unknown.

Corroborating this is an animal study in which the solitary tract nucleus was electrically stimulated directly, resulting in increased cerebral blood flow as well as enhanced synchronization of the electroencephalogram[31]. And another animal study showed that vagus nerve stimulation significantly reduces the size of induced brain infarction by an unknown mechanism which is nonetheless independent of increased cerebral blood flow[32], thereby implying an additional mechanism of neural protection. In light of these findings it may not be surprising that direct electrical stimulation of the cerebral cortex[33] in animals protects that cortex from induced ischemia by mechanisms that are antiapoptotic, angiogenic, and anti-inflammatory. But more important to brain dentistry may be the indirect stimulation of a specific area of brain tissue by functionally stimulating peripheral nerves that lead to that specific area; and animal studies have shown this to significantly reduce the size of induced infarctions[34].

MOLAR MODULE

By the year 2050 "molar modules" may be among the most widely used dental appliances for brain dentistry in adults, due in part to the relatively large size of molars, as well as their posterior position in the mouth. An implanted molar module might consist of a permanent intraosseous metallic hardware casing which could hold computer chips and/or software that could be removed, updated, and replaced *via* a removable extraosseous dental crown. The site of implantation on the alveolar ridge would maintain the integrity of the dental arch and be consistent with overall dental health and dental occlusion. The molar module might fill a space previously made available by the extraction of teeth. Although there has been no previous experience using molar modules or brain dentistry in either animal models or humans, their usefulness is very plausible.

CHORDA TYMPANI NERVE

Brain dentistry may utilize computerized electronic stimulation of the chorda tympani nerve *via* a submucosal wire from the molar module to a site where the chorda tympani nerve runs together with the lingual nerve. This is a site frequently used by dentists for the blind injection of local anesthetic agents to anesthetize one side of the tongue together with the adjacent gingiva when performing procedures on the mandibular teeth. The chorda tympani nerve mediates taste sensation from the anterior 2/3 of the tongue, and is largely an innocent bystander for purposes of the dental injection.

Stimulation of the chorda tympani nerve indirectly stimulates the rostral third of the solitary tract nucleus of the medulla *via* functional connections, just as vagus nerve stimulation indirectly stimulates the caudal 2/3 of the solitary tract nucleus. Stimulation of the chorda tympani nerve would also indirectly stimulate the superior salivatory nucleus of the medulla *via* fibers which innervate the submandibular and sublingual glands in the floor of the mouth. Both vagus nerve stimulation and chorda tympani nerve stimulation may ultimately be utilized as treatment modalities in medullary neurology.

The development of wireless communication between the molar module and a secondary transmitter placed at the nerve site would minimize the invasiveness of the procedure to place a secondary transmitter and might open the possibility of blind transmucosal or percutaneous injection of a very small secondary transmitter, inasmuch as a wire connecting it to the molar module would be unnecessary.

Molar modules might eventually be multifunctional with roles in both monitoring and therapy. Cardiac rhythm and local tissue chemistries might be recorded and transmitted to a remote data base or mobile device. Synchronized stimulation of the auditory nerve by sub-audible sound pulses would indirectly stimulate the cochlear nucleus in the medulla. Future research will determine the value of these potential therapeutic modalities and others[35-37]. Some of the potential general goals of brain dentistry are to provide neural protection to vital centers of cardiac and respiratory function and to enhance their performance by optimizing blood flow, preventing ischemic injury, and enhancing electrical stability.

RECENT RELEVANT RESEARCH

Firstly, there have been no research publications regarding brain dentistry specifically - but medical investigators have recently been studying ways to prevent sudden deaths in vulnerable patient groups that have relevance to events in the medullary autonomic nuclei. The results of an international trial studying prevention of sudden death and near-death cardiac events in 1325 patients who had heart failure with central sleep apnea[38] have recently been reported. In this trial the patients were given a treatment which augmented pulmonary ventilation nocturnally and presumably improved systemic blood gasses nocturnally. The study, however, ignored the issues of neuro-electrical instability in the medullary autonomic nuclei, and it ultimately concluded that the ventilatory assistance given to the patients did not improve their outcomes, but in fact worsened them[38].

An alternative therapy under investigation is phrenic nerve stimulation. A recent international trial of only 57 patients with central sleep apnea who received unilateral phrenic nerve stimulation[39] showed that it was both safe and effective, even for those who also had heart failure. Although primarily a motor nerve (to the diaphragm), the phrenic nerve carries both sensory and autonomic fibers as well. Phrenic nerve stimulation may possibly have a modulating effect on medullary autonomic nuclei, which might help to prevent adverse cardiac events and improve patient outcomes.

Lastly, to the extent that vagus nerve stimulation is a

proposed preventive therapy for sudden unexpected death related to medullary brain lesions, it might be expected to reduce or eliminate the incidence of sudden unexpected death in epilepsy in the intractable epilepsy patients who receive it as treatment. But a recently published study[40] of 466 patients showed that vagus nerve stimulation did not influence the incidence of sudden unexpected death, although its data were collected between 1995 and 2010 and reflected the technology and methods of that time-frame[40]. Other investigators[41] of vagus nerve stimulation in epilepsy patients, however, have reported a favorable cardiac electrical stabilization, which encourages further research. And only very limited research has been done regarding the specific electronic parameters for stimulation of either the vagus or phrenic nerves, and this could greatly influence outcomes. Much more research is needed.

CONCLUSION

Vagus nerve stimulation has been used with relative safety and effectiveness to treat epilepsy, whereby it is thought to stabilize electrical activity in the brain and has been shown to increase blood flow in the medulla. Vagus nerve stimulation indirectly stimulates the solitary tract nucleus, an autonomic site most often involved in cases of sudden unexpected death related to medullary brain lesions, and where blood flow and electrical stability have been called into question.

It is plausible that vagus nerve stimulation might be used in the prevention of sudden unexpected death related to medullary brain lesions as well as non-fatal symptoms, such as "chest pain from the brain", possibly caused by ischemia and electrical instability in medullary autonomic nuclei. In future years, electrical stimulation of the chorda tympani nerve may be used to indirectly stimulate the solitary tract nucleus with the same goals.

A vast frontier within a small space, the medulla oblongata will likely be the focus of some of the most significant medical advances of the 21st century. Brain dentistry will probably play an important role in both monitoring and therapy related to the brain in general as it accompanies the development of medullary neurology.

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REVIEW

Time windows for postnatal changes in morphology and membrane excitability of genioglossal and oculomotor motoneurons

Livia Carrascal, JoséLuis Nieto-González, Ricardo Pardillo-Díaz, Rosario Pásaro, Germán Barrionuevo, Blas Torres, William E Cameron, Pedro Núñez-Abades

Livia Carrascal, JoséLuis Nieto-González, Ricardo Pardillo-Díaz, Rosario Pásaro, Blas Torres, Pedro Núñez-Abades, Department of Physiology, University of Seville, 41012 Seville, Spain

Germán Barrionuevo, Department of Neurocience, University of Pittsburgh, Pittsburgh, PA 15260, United States

William E Cameron, Department of Behavioral Neuroscience, Oregon Health and Science University, Portland, OR 97239, United States

Pedro Núñez-Abades, Departamento de Fisiología, Facultad de Farmacia, Universidad de Sevilla, 41012 Sevilla, Spain

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Correspondence to: Pedro Núñez-Abades, PhD, Departamento de Fisiología, Facultad de Farmacia, Universidad de Sevilla, C/ Profesor García González nº2, 41012 Sevilla, Spain. pnunez@us.es
Telephone: +34-954-556130

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Abstract

Time windows for postnatal changes in morphology and membrane excitability of genioglossal (GG) and oculomotor (OCM) motoneurons (MNs) are yet to be fully described. Analysis of data on brain slices *in vitro* of the 2 populations of MNs point to a well-defined developmental program that progresses with common age-related changes characterized by: (1) increase of dendritic surface along with length and reshaping of dendritic tree complexity; (2) disappearance of gap junctions early in development; (3) decrease of membrane passive properties, such as input resistance and time constant, together with an increase in the number of cells displaying sag, and modifications in rheobase; (4) action potential shortening and afterhyperpolarization; and (5) an increase in gain and maximum firing frequency. These modifications take place at different time windows for each motoneuronal population. In GG MNs, active membrane properties change mainly during the first postnatal week, passive membrane properties in the second week, and dendritic increasing length and size in the third week of development. In OCM MNs, changes in passive membrane properties and growth of dendritic size take place during the first postnatal week, while active membrane properties and rheobase change during the second and third weeks of development. The sequential order of changes is inverted between active and passive membrane properties, and growth in size does not temporally coincide for both motoneuron populations. These findings are discussed on the basis of environmental cues related to maturation of the respiratory and OCM systems.

Key words: Development; Motoneurons; Respiratory system; Oculomotor system; Neuronal plasticity

Core tip: For more than 2 decades, numerous studies have tried to describe time windows of changes of membrane properties of motoneurons. This review aims to show what mechanisms are implied in those changes as well as how they are triggered. Our findings are focused on genioglossal and oculomotor motoneurons from birth to adult age. The perspective adopted is the description of how those changes correlate with both intrinsic and extrinsic factors. Data in this review is relevant to understand pathologies related to development.

Carrascal L, Nieto-González J, Pardillo-Díaz R, Pásaro R, Barrionuevo G, Torres B, Cameron WE, Núñez-Abades P. Time windows for postnatal changes in morphology and membrane excitability of genioglossal and oculomotor motoneurons. *World J Neurol* 2015; 5(4): 113-131 Available from: URL: http://www.wjgnet.com/2218-6212/full/v5/i4/113.htm DOI: http://dx.doi.org/10.5316/wjn.v5.i4.113

INTRODUCTION

For the past 20 years, our lab has carried out research in order to understand how the anatomical and electrophysiological properties of MNs may change during postnatal development. Our studies have included analyses of rat MNs during their first month of life, from birth to day 30, when they become young adults. Our work has focused on respiratory MNs of the hypoglossal nucleus (genioglossal subpopulation, GG)[1-8], as well as of the oculomotor (OCM) nucleus[9-13]. The genioglossus is innervated by the ventromedial section of the hypoglossal nucleus[14]. The genioglossus is responsible for tongue protrusion and is activated before the diaphragm during respiration in order to keep the upper airway open[15]. MNs within the OCM nucleus innervate extra-ocular muscles[16] and drive eye movements[17-19]. Our studies have incorporated *in vitro* techniques with brain slices that can keep the tissue alive for hours. In these slices, it is possible to perform intracellular recording techniques and simultaneous labeling that allow for the morphological analysis of the cells recorded[1,3,5,7,9-11,13,20]. In this *in vitro* preparation the ionic setting can be easily controlled while the neurotransmitters and the drugs are being added to the extracellular medium[2,6,12,21-24]. Our results reveal that both pools of MNs undergo important modifications in their morphological and physiological characteristics during postnatal development. These changes are likely to start in embryologic stages and that what we are showing is the achievement of the typical characteristics of the adult phenotype[25-27].

For the purpose of comparison, data from GG and OCM MNs were grouped in P1-P5, P6-P10, P11-P15, and P21-P30[1-13]. A detailed comparison of the results obtained could provide information on: (1) the existence of a common maturation sequence in mammalian MNs; (2) the description of the progression of the physiological and anatomical features to reach an adult stage; (3) the different strategies and time windows that are used in the sequence of maturation; and (4) the causes behind all these phenomena. The identification of the time windows in which changes of morphological and electrophysiological properties take place would make these MNs a suitable model in order to study the mechanisms and molecules that are involved in such changes. To understand the existence of time windows of changes, we must keep in mind that the adult neuronal phenotype is shaped by a combination of genetic and epigenetic features[28]. Therefore, some of the changes that we present could also be a consequence of internal watches[29], or changes in trophic factors and/or synaptic inputs converging on MNs with age, or probably due to modifications that affect the properties of target muscle fiber composition[30-33].

DENDRITIC TREES MODIFY THEIR COMPLEXITY DURING POSTNATAL DEVELOPMENT

Dendrite morphogenesis involves an active and complex process of formation, maintenance or elimination of dendritic branches[34-37]. We have observed that the number of primary dendrites per MN is approximately 6, for the GG MNs, and 5 in the case of OCM MNs, numbers that remain unchanged with age[5,11]. However, the tree is largely remodeled during development. Dendritic trees of GG MNs are equally complex at birth and in the adult age, but they go through a stage of transient simplification (reduced complexity around P15)[5] during development. The dendritic complexity observed at birth gradually decreases during the first ten days in the number of branches per neuron, branch order, and number of terminal branches per neuron (Figure 1A-C). The elimination of dendritic branches (with a complete loss of the 6th-8th order branches) tends to increase the symmetry of the tree (schematically represented in Figure 2C). Then dendrites recover at P21-30 in the adult, exhibiting a similar complexity level as the one found in the newborn. This pattern of postnatal maturation in dendritic complexity was also described for spinal MNs[38,39]. However, a postnatal simplification of the dendritic tree may not be a general principle governing postnatal maturation of MNs, as proposed by Nuñez-Abades *et al*[5]. A different temporal pattern of dendritic complexity was later found in OCM MNs (Figure 1A-C). During the first stage (up to P10), the number of first order dendrites did not change, but the number of branches constantly increased as being observed in the number of branches per neuron, the branch order and the number of terminals per neuron (Figure 1A-C). During the second stage, the number of

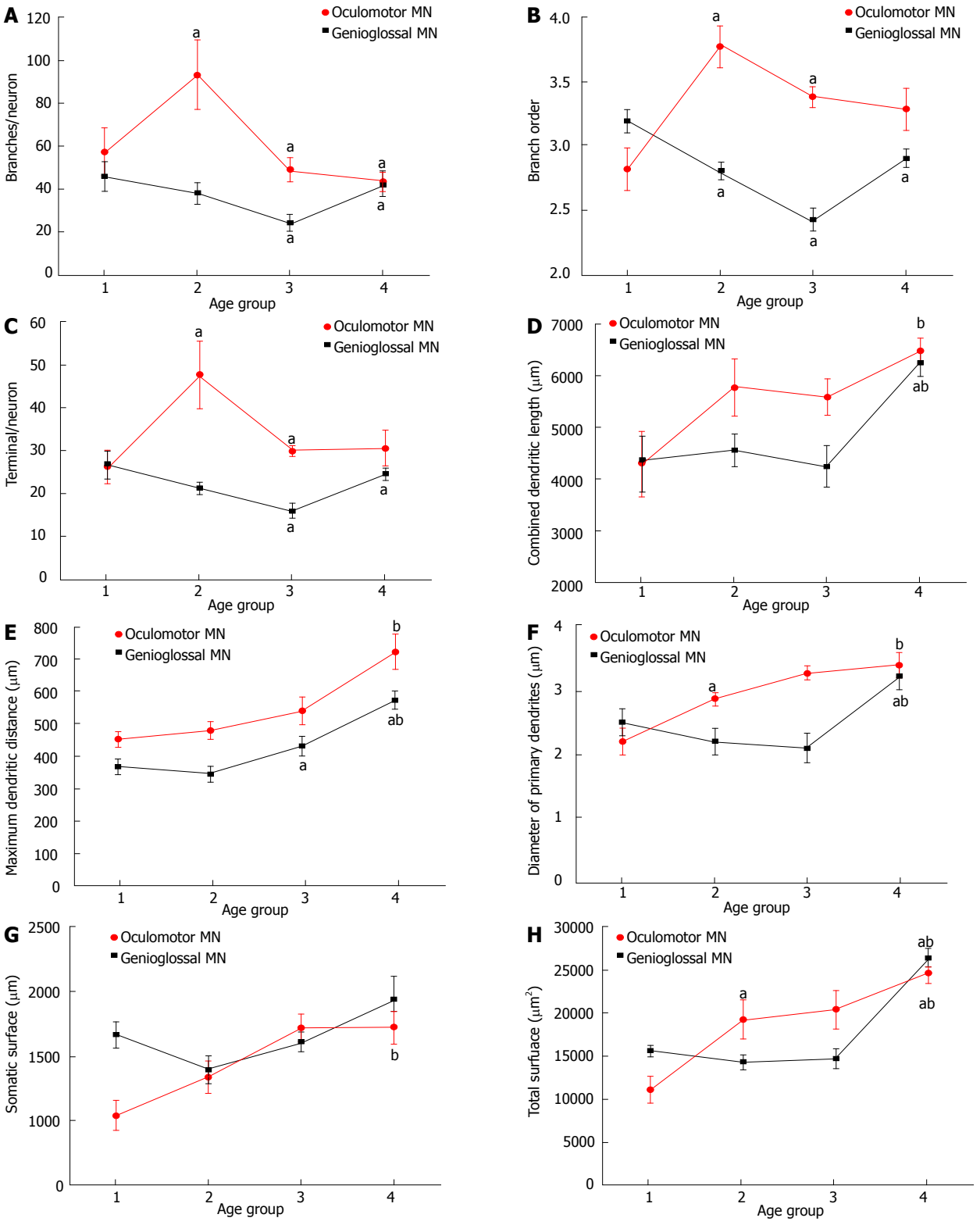

Figure 1 Postnatal maturation of morphological properties of motoneurons from genioglossal and oculomotor nuclei. A-C: Changes in dendritic complexity measured as number of branches per neuron (A), branch order (B) and number of terminals per neuron (C). Note that in GG MNs complexity decreases up to P15 and then increases, while in OCM MNs there is an increase up to P10 and then decreases. The 2 opposite development strategies lead to the same outcome in the adult rat when compared with initial values; D and E: Changes in neuronal length measured as combined dendritic length (D) and maximum distance from soma to dendritic terminal (E). Note in D-E that dendritic length progressively increases for both populations of MNs with the most relevant changes at the late stages of development; F and H: Changes in neuronal size measured as diameter of primary dendrites (F), somatic surface (G) and total surface area (H). Note that in F and H OCM MNs grow between P1 and P10, while GG MNs increase mainly between P15 and P30. OCM MNs show a slow growth of the somatic surface along development, while it is already established at birth in the case of GG MNs. In this figure and the following: (1) the plots illustrate mean values for each parameter and age for OCM (red circle) and GG (black square) MNs; (2) measures are expressed in mean ± standard error; (3) the "a" indicates statistical significance between two consecutive age groups; and (4) the "b" represents statistical significance between age group 1 and age group 4. The age groups 1, 2, 3 and 4 correspond to P1-P5, P6-P10, P11-P15 and P21-P30, respectively. The data from GG[5,7] and OCM[11] come from previous studies. MNs: Motoneurons; GG: Genioglossal; OCM: Oculomotor.

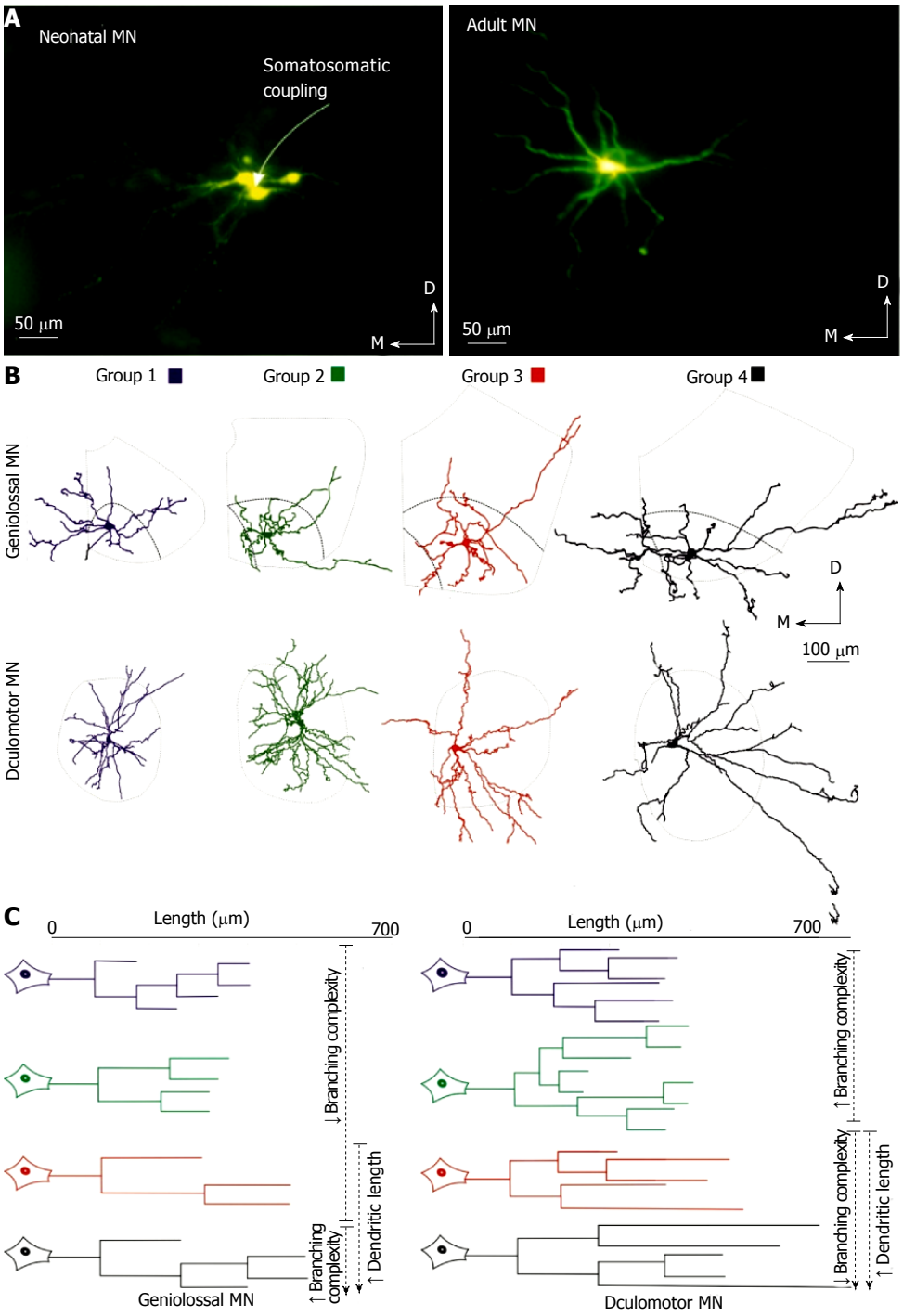

Figure 2 Summary of the main morphological changes of motoneurons from genioglossal and oculomotor nuclei. A: Photomicrographs of representative GG MNs injected intracellularly with Lucifer yellow in a newborn (left image) and juvenile adult rat (right image). Note the presence of coupling between neonatal MNs. Only one cell was injected, however 3-4 are labeled; B: Changes in nucleus size and dendritic arborization orientation of MNs during postnatal development. It is remarkable that dendrites in the younger MNs are restricted to the nucleus boundaires, whereas the 2 oldest groups show some portions of dendrites outside those limits, preferably in the ventrolateral axis. The outer line indicates the boundary of the nuclei, and the inner line shows the limits of the GG nucleus; C: Dendrograms for a representative dendrite for each age group of GG (left) and OCM (right) MNs. Note the growth in length for both groups of MNs and the changes in dendrite architecture that take place during development. The data from GG[3,5] and OCM[11] come from previous studies. MNs: Motoneurons; GG: Genioglossal; OCM: Oculomotor.

branches went down to newborn values[11]. The results described above may indicate that there is not a single maturational pattern of dendritic complexity in MNs. However, the observed changes in dendritic complexity may underlie a strategy that allows for dendritic elongation, as reported by Cameron *et al*[38].

DENDRITES ELONGATE DURING POSTNATAL DEVELOPMENT

The enlargement of dendrites during postnatal development is a common characteristic for every pool of MNs studied, including brainstem and spinal MNs of

different species[5,11,38-42]. In GG MNs, dendritic combined length is maintained from birth to day 15 (Figure 1D) and they show an evident tendency to increase in maximum dendritic length (Figure 1E). Later, between P15 and P21-30, this proliferation of branches, that occurs in the intermediate parts of terminal branches[5], significantly augment the combination of dendritic length and maximal distance (Figure 1D and E). In accordance with that data, sholl diagrams evidence how adult MNs exhibit dendrites about two-fold larger than those stated around P10 in OCM nucleus MN[11]. In OCM MNs, therefore, dendritic elongation takes place between P10 and P21-30, in maximal dendritic distance (Figures 1E and 2C), and gradually from birth to P21-30 in dendritic combined length (Figures 1F and 2C). An interesting aspect is to know whether neurons elongate in preferential directions to establish the adult territories.

The lengthening of neonatal mouse lumbar MNs during the first 2 week after birth can be compared with the growth of the spinal cord[40]. However, the remarkable growth in length of GG and OCM dendrites is bigger than the size the nuclei increase. Then, dendrites can be found outside the boundaries of the GG and OCM nuclei in adult MNs in a area larger and in a higher percentage than those for newborn MNs (Figure 2B). Studies carried out on adult rat hypoglossal MNs[43] indicate the existence of extranuclear dendrites that are distributed along four separate areas, including the nucleus of tractus solitaries, the nucleus raphe obscurus, the medullary reticular formation, and the contralateral hypoglossal nucleus. We have found that these dendritic domains are not established at birth but rather their proliferation shows up during development[5]. Two and three-dimensional analyses showed a new tree configuration for GG MNs from birth up to days 5-6 consisting of the resorption of dendrites in the medial, dorsal, and dorsomedial directions (Figure 2B). Dendrite growth expands in all directions between days 13-15 and 19-30, but with a greater increase in the medial and lateral sectors (Figure 2B), and dendrites are now placed outside the nucleus, mostly oriented towards the dorsolateral and ventrolateral regions, particularly in the adult age. As described for the GG nucleus, dendrites of OCM MNs of adult rats extend outside the nucleus in a larger area and in a higher percentage than those found in newborn MNs (Figure 2B). Dendrites in the transverse section for P11-P15 and adult MNs, are mainly oriented outside the nucleus in the dorsal and ventrolateral directions.

An interesting question that arises is how dendritic fields and the boundaries of dendritic fields are established[36,44]. We must bear in mind that dendrites of the GG and OCM MNs remain inside the boundaries of their nuclei from birth to P10 (Figure 2B) with clear signs of dendritic retractions that keep them inside the nuclei displaying a round-shaped distribution. It is known that the repulsive dendro-dendritic contacts determine the basis of contact-mediated inhibition of dendritic growth[45]. Then, our findings might suggest that those dendro-dendritic interactions also constitute a regulation element for GG and OCM MNs in the first 10 d of life. Another question is how the direction of dendrite out-growth is determined. A large number of transcription factors regulate various aspects of dendrite development[36,46]. However, the way in which they regulate the size and the pattern of dendritic fields in order to produce the MN identity is not yet fully understood[47-51]. Furthermore, local signals activate processes of arborization and elongation of the growth cones that are located in the terminal branches of the dendritic trees[36,46,52,53]. Extrinsic signals, such as neurotrophins, and neuronal activity seem to induce dendrite development modifying the organization and dynamics of the cytoskeleton[36,54-57]. We have found that GG and OCM MNs show a differential enlargement of dendrites in some orientations from P10 to P21. Then, it can be proposed that dendrites in GG and OCM MNs respond unevenly to extracellular cues, suggesting that they are asymmetrically distributed in different dendrites. Furthermore, the observed dendritic growth in particular time windows may be related to the arrival of new synaptic inputs[35]. In GG MNs, dendrites that extend dorsolaterally into the tractus solitaries between P15-P30 may synaptically connect from peripheral respiratory receptors[58] that are important to induce hypoglossal reflexes (*i.e.*, swallowing)[59]. Dendrites extending laterally could be targeted by trigeminal afferents that are related with coordination of the tongue and jaw[60], and dendrites orientated ventrally could be the target of innervation from nucleus raphe obscurus, related to state-dependent activity (*e.g.*, sleep/wake, rest/exercise)[61] and mediating CO_2 chemosensitivity[62] or from excitatory and inhibitory respiratory premotor neurons[30,63-69]. The ventrally oriented dendrites in OCM MNs in P15-P30 would display a location closer to the medial longitudinal fasciculus. These vestibular afferents elicit eye movements at around P21[70-71]. In addition, the elongation and simplification of dentritic trees with postnatal development may underlie the stratification of different synaptic inputs[72]. and, in OCM MNs, they may provide a means for the separate control of visuomotor and vestibular functions[73].

POSTNATAL DENDRITIC RESHAPING GOES ALONG WITH GAP JUNCTION WITHDRAWAL

Gap junctions couple MNs at the embryonic and the early postnatal periods[25]. This coupling is present in newborn GG MNs up to 8 d after birth as demonstrated by intracellular injection with Lucyfer yellow (Figure 2A)[3]. A similar finding was found in OCM MNs[11]. The loss of electronic coupling in MNs with age is allows for the acquisition of individual motor units[74]. While present in early postnatal stages, gap junctions contribute to

synchronous firing[75] and, likewise they could help to synchronize collective discharge in GG MNs. This has the immediate effect of producing a strong and uniform tongue protusion that is required in important motor tasks (such as sucking, breathing, and swallowing) from the moment the animal is born[3]. However, it is not easy to extend this hypothesis to ocular MNs, since the latter are only ready in P21. This is the precise moment when eye movements are performed as a result of the visual and vestibular stimuli[70,71]. Another hypothesis is that this early coupling before P11 helps to establish the necessary "prewiring" for the progressive formation of neural circuits[76]. When gap junctions are removed the polyneuronal innervation of muscle fibers is also eliminated in a process that seems to be under the control of trophic factors[77,78]. In fact, the timing for that disappearance may be disrupted when the muscle is paralized in the neonatal rat, demonstrating that trophic factors arising from the target muscle are needed to maintain gap junctions in MNs[79]. Thus we propose that coupling in GG and OCM MNs is removed when polyneuronal innervation has also disappeared in the muscles that they innervate, and when prewiring of neuronal circuits on those MNs has been established.

POSTNATAL INCREASE IN SIZE IS NOT A CONTINUOUS PROCESS

Somatic and dendritic neuronal size is not established at birth[38]. In a pioneer study, researchers found that the growth in membrane surface area of developing spinal MNs of the cat can be considered a continuous process[80,81]. However, our investigations of phrenic MNs in the cat[38], as well as in GG MNs of the rat[7] disagree with that hypothesis. In fact, GG MNs (from birth to P15) were characterized by a lack of growth in dendritic diameter and dendritic surface area (Figure 1F-H). In this time window, maturation results in more surface area being placed at distances farther away from soma by the redistribution of the preexisting membrane (Figure 2C)[7]. Later, beyond day P15, the dendritic surface area doubles because of the generation of new terminal branches and the increase in dendritic diameter at all branch orders (especially significant was the increase in the 1st order diameter, see Figure 1F). Growth in diameter in cat spinal MNs has also been reported to occur late in development[38,39]. However, an earlier time window for growth in dendritic size was found in OCM MNs. In these MNs, dendrites increase exponentially until around P10 (Figure 1F-H)[11]. In this period, the membrane area of dendritic trees increases by a greater arborization. Later (beyond P10), maturation produces a greater area farther away from the soma, by the use of the pre-existing membrane, but at the expense of lowering the complexity of the dendritic arborization (Figure 2D). Despite growth observed in somal dimensions in OCM MNs measured postnatally (Figure 1G)[7,11], the dendritic to somal surface area ratio increases postnatally in GG and OCM MNs, as concluded for other similar studies on developing spinal MNs[38,39,81]. In general, it seems that there is more dendritic surface area available for afferent synapses in developing MNs than in newborn MNs.

A DECREASE IN TIME CONSTANT AND INPUT RESISTANCE CHARACTERIZES DEVELOPMENT

In Figure 3A we illustrate that the same current amplitude evokes a larger hyperpolarization in the youngest MN, which implies a larger input resistance when compared with the adult. Input resistance was about 50% less in GG motoneurons and about 25% less in OCM MNs (Figure 3B and C)[4]. This reduction is common to different pools of MNs[82-85]. Figure 3C depicts how the drop in resistance for GG MNs happens in a narrow time window between P10-P15, while the same drop takes place between P5-P10 in the case of OCM MNs.

As seen in Figure 3A, in newborn GG MNs (and also in OCM MNs, not shown), the voltage response to current negative pulses approaches a steady-state level exponentially. Furthermore, the relationship between current negative pulses and voltage response is almost linear (Figure 3B). As a response to negative current steps, adult GG MNs present a membrane potential rectification that is characterized by a depolarizing drift or "sag" (Figure 3A)[4]. This physiological phenomenon has also been reported in other motoneuronal pools[84,86-88]. The frequency of sag increases ten times gradually in GG and OCM MNs, without the appearance of a clear time window for changes[4,10]. An inward rectification current (Ih) is believed to be underlying this sag. This current is largely carried by sodium ions and can be blocked by extracellular cesium[2,89] and may participate in the postinhibitory rebound seen in the adult MN as illustrated in Figure 3A. The increasing frequency of sag with age is paralell to a bigger density of channels carrying Ih current (Figure 4)[90]. Although Ih current is half-active at rest, as demonstrated by voltage-clamp experiments[89,90], it is unlikely that this conductance underlie the decrease in input resistance during development[1,91].

If we accept that specific membrane capacitance stays unchanged during development, the decrease in time constant in MNs would be a consequence of the reduction in the specific membrane resistance[85]. In Figure 3D, we illustrate how the time constant in GG and OCM MNs falls around 40% and 30%, respectively, in the time window found for the decrease in input resistance. This coincidence in time framing points to one mechanism that can explain both phenomena: A decrease in specific membrane resistance. In summary, the decrease in time constant, input resistance, and probably specific membrane resistance must be genetically programmed during postnatal development for all MNs, and it is even observable in MNs in culture[29].

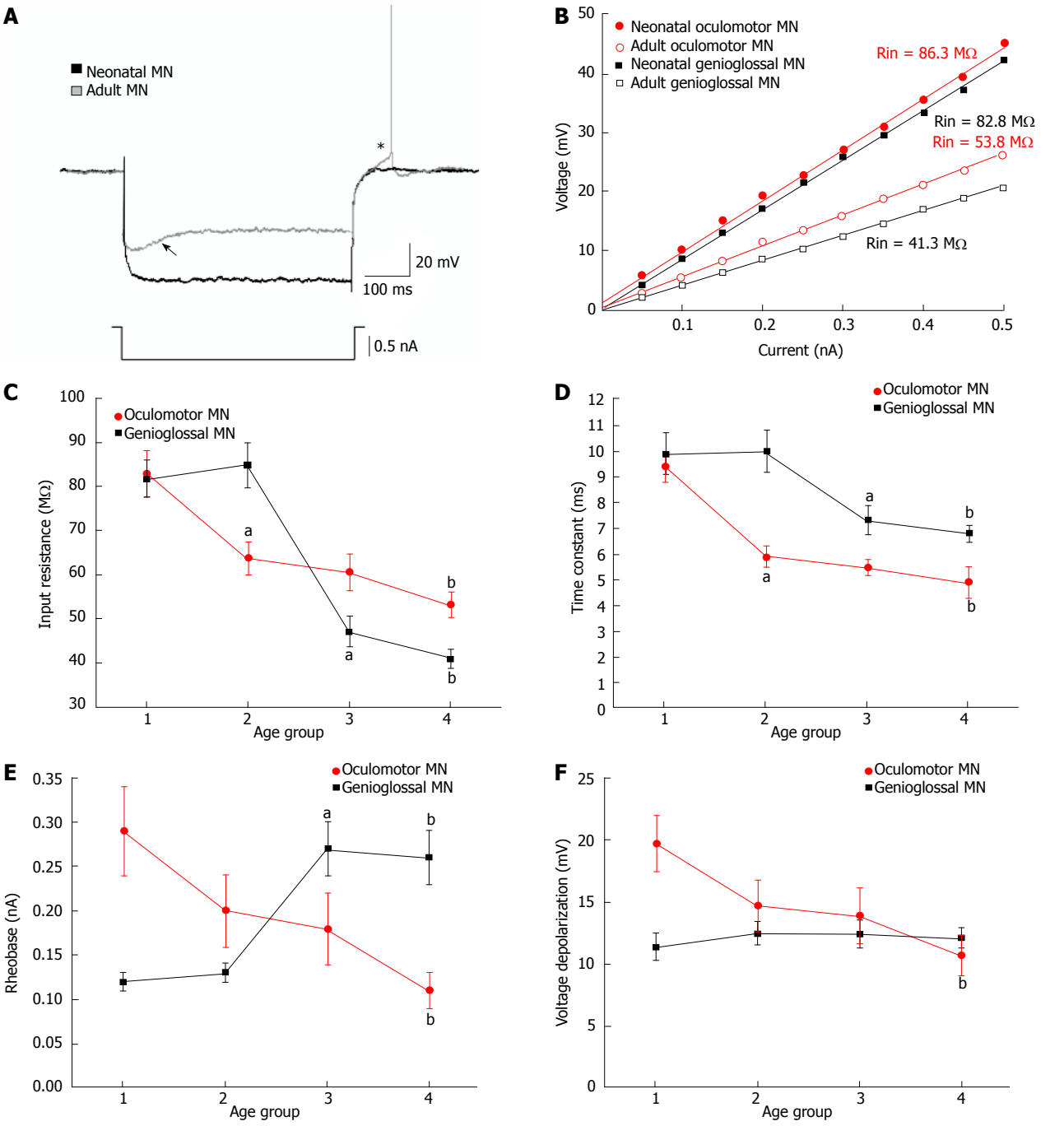

Figure 3 Postnatal maturation of passive membrane properties of motoneurons from genioglossal and oculomotor nuclei. A: Voltage membrane response for one representative GG neonatal MN and one representative adult MN to negative current pulses of 0.8 nA. As shown, the same current amplitude of current evokes a larger hyperpolarization in the youngest MN. Also note the presence of sag (see arrow) and postinhibitory rebound (asterisk) in the adult MN but not in the neonatal one; B: Relationship between current intensity and voltage response in neonatal and adult MNs: Neonatal OCM MN (filled red circle); adult OCM MN (open red circle); neonatal GG MN (filled black square); adult GG MN (open black square). The slope of the relationship determines input resistance. Note that, for both populations of MNs, input resistance decreases with development although this decrement is bigger for GG MNs; C-F: Plots illustrating changes on input resistance (C), time constant (D), rheobase (E) and voltage depolarization (F) during postnatal development for GG and OCM MNs. Input resistance and time constant decrease during development in both populations, while rheobase increases in GG MNs and decreases in OCM MNs. The "a" indicates statistical significance between two consecutive age groups; and the "b" represents statistical significance between age group 1 and age group 4. The age groups 1,2,3 and 4 correspond to P1-P5, P6-P10, P11-P15 and P21-P30, respectively. The data from GG[4] and OCM[10] come from previous studies. MNs: Motoneurons; GG: Genioglossal; OCM: Oculomotor.

However, the differential time window of changes in passive membrane properties for each population of MNs would lead to the conclusion that extrinsic factors trigger the onset of the change, revealing that each population might be specialized depending on their function.

The drop in specific membrane resistance within postnatal development could be attributed to a larger membrane surface[92]. Our data demonstrate a significant correlation between total membrane surface and input resistance in newborn and in adult OCM MNs[13]. However, when we combine neonatal and adult MNs

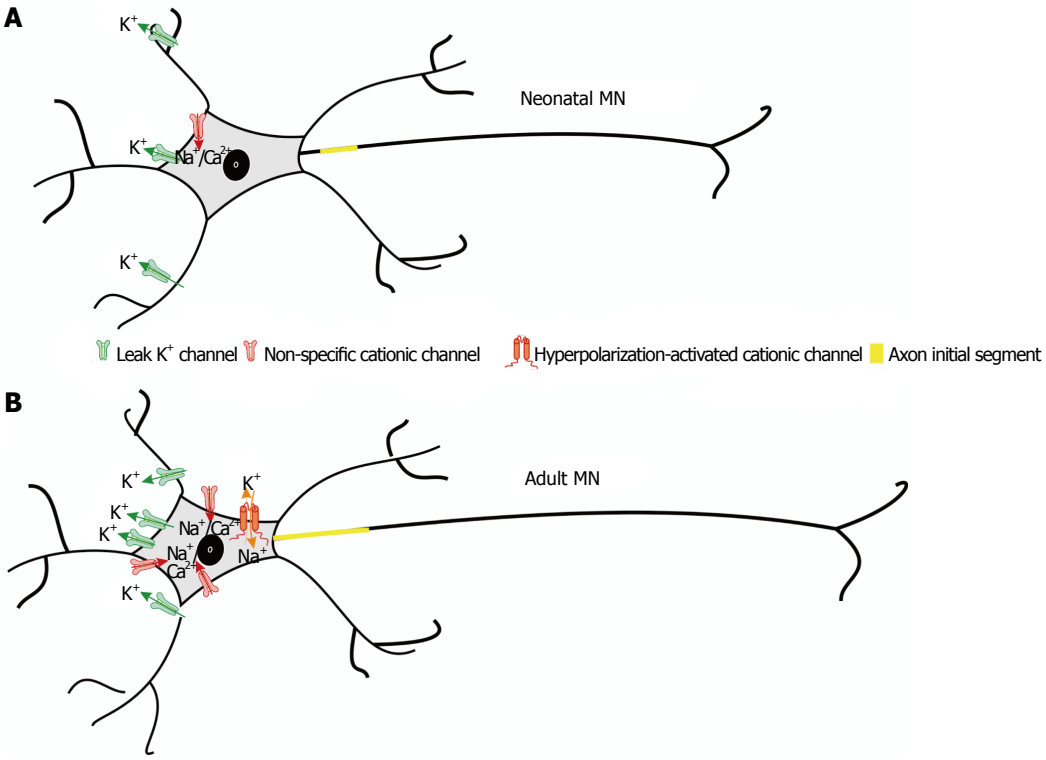

Figure 4 Hypothesis of mechanisms underlying input resistance and rheobase modifications during development. Schematic drawings illustrating proposed differences in ion channels (number, type and distribution) and axon initial segment (length and proximity to soma), between neonatal (A) and adult (B) MNs. Note that, in the adult MN, the axon initial segment is represented larger and closer to soma. Besides, in the neonatal MN, leak potassium channels are located more distal to the soma and in a less number. Hyperpolarization-activated cationic channels responsible to the sag are only present in the adult MN, with a high number of non-specific cationic channels responsible for the persistent calcium and sodium currents. MNs: Motoneurons.

in a single group, size and input resistance do not correlate well[8,13]. This disagreement could be explained by the fact that changes in input resistance and size are not linked, as demonstrated in spinal MNs[93].

The proliferation of tonically active synaptic inputs was used to analyze the postnatal decrease in the passive membrane properties in rat GG MNs. Both hypoglossal and OCM MNs may be receiving GABA, glycine, and glutamatergic synaptic inputs that may be tonically active[23,24,94,95]. Then, developmental alterations in the number or kinetics of the neurotransmitter receptors may produce some of the electrophysiological observable changes[6,95-97]. The role of the synaptic input was evaluated by the selective blockage of neurotransmitter release associated with action potentials, calcium-dependent and calcium-independent release in developing GG MNs[6]. From these experiments we concluded that: (1) synaptic input contributes to the resting conductance of the MN membrane under development; (2) the role that glycine/GABAA receptors may play to determine resistance becomes dominant in the adult, suggesting a proliferation of inhibitory synaptic inputs with age; and (3) the proliferation of synaptic inputs is not enough to explain the large decrease in input resistance occurring between days P10 and P15 in developing GG MNs. In OCM MNs, it seems that GABA, but not glutamate, may contribute to membrane resistance in juvenile rats[23,24]. On the other hand, noradrenergic and serotoninergic modulation in hypoglossal MNs has also been associated with significant changes in neuronal input resistance[97,98].

Second, we have studied whether a possible proliferation of K^+ channels during postnatal development could be the reason for the decrease in input resistance in GG MNs between P10-P15[2]. The addition of a potassium channel blocker, tetraethylammonium, to the extracellular medium in the presence of high magnesium largely increases both input resistance and time constant, indicating a major role for K^+ channels that are not related to synaptic transmission. More drastic changes occur when external barium is applied, known to be able to block the "leak" K^+ channel[99]. An additional manipulation of K^+ channels was obtained by the intracellular injection of cesium[2]. Thus, from these experiments it was concluded that cells with a low resistance have a greater number of cesium- and barium-sensitive channels than cells with a high resistance. Then, the current hypothesis (Figure 4) suggests that the main factor to explain the fall in passive membrane properties is probably an increase in the expression of a leak K^+ current over development, that is partly mediated by TASK-1 and TASK-1/3 heteromeric channels[100,101], between P10-P15 in GG that can be extended to OCM MNs[102]. Furthermore, both anatomical evidence[101] and the data obtained with a model for sympathetic neurons[103] applied to GG MNs[1] support a differential distribution of leak K^+ channels in dendrites. We

propose that the potassium conductances more distally located at birth are probably uniformly redistributed across the adult MN membrane (Figure 4)[1,2].

CHANGES IN SPECIFIC MEMBRANE RESISTANCE WOULD LEAD TO PHYSIOLOGICAL ALTERATIONS IN MOTONEURON EXCITABILITY DURING POSTNATAL DEVELOPMENT

The minimum injected current required to elicit an action potential (rheobase) is a measure of cell excitability[4,9]. Considering that the amount of depolarization required to reach threshold in GG MNs does not change with age (Figure 3F), the 2 times increase found in rheobase during postnatal development in these MNs must be the result of a decrease in specific resistance (Figure 3E). Supporting this conclusion is the fact that the increase in rheobase happens in a time window identical to the one shown by the decrease of the input resistance. This finding suggests that the membrane of the GG MNs behave as an ohmic membrane. By contrast, in OCM MNs, we found a gradual decrease in the rheobase with age (Figure 3E)[10] as found in cortical pyramidal cells with age[104]. These results may suggest that the postnatal development in rheobase depends on the population of MNs. In OCM MNs, the decrease in the rheobase (Figure 3E) goes along a decrease in the depolarization voltage needed to reach threshold (Figure 3F). Thus, in these MNs, the excitability within the nucleus increases in spite of the lower membrane resistance[9]. The drop of the voltage threshold with age in OCM nucleus MNs could motivated by an increase in long lasting Ca^{2+} currents and persistent Na^{+} conductance. These inward currents, which are activated at the subthreshold level, may produce excitation and be implied in MN recruitment[105-109]. Several studies report about these conductances in brainstem and spinal MNs[84,88,110,111]. If these currents actually increase during postnatal maturation[112], thus influencing spike threshold, they might explain the decreased depolarization voltage in adult OCM nucleus MNs. We propose that these currents are increased gradually during postnatal maturation (Figure 4), but their influence on action potential generation differs between the two pools. Different explanations could be given to explain the diminution of voltage threshold at postnatal age in OCM MNs. For instance, new findings have shown plasticity in the axonal initial segment that influences cell excitability[113]. A possible hypothesis is how synaptic deprivation or chronic depolarization can modify the location and extent of this spike triggering zone[114-116]. The enlargement of the axon initial segment, which goes along bigger voltage-gated sodium currents, decreases both the current and voltage threshold to trigger action potential[116]. The same can be proposed in OCM MNs during development (Figure 4). Extended studies on the modifications in ionic currents (voltage gated sodium current, persistent inward current, *etc.*) and in the axon initial segment should provide further insight to interpret the physiological bases of changes in the rheobase and voltage threshold during development. We have demonstrated that an active membrane property, namely voltage threshold, not associated with cell size - input resistance, is the one that determines the recruitment order of MNs during postnatal development[13]. Furthermore, we have also reported that voltage threshold is modulated by acetylcholine in OCM MNs[21] and this modulation is enhanced with age[12].

CHANGES IN ACTION POTENTIAL PROPERTIES WITH AGE

GG and OCM MNs undergo several changes in their action potential properties, and the subsequent medium afterhyperpolarization (mAHP), during the first 3 wk of life (Figure 5A-D). The first week is characterized by a decrease in the duration of the action potential and the mAHP in developing GG MNs. Even though no change can be observed in the resting membrane potential or action potential height during development, the action potential is shortened as a result of more rapid depolarization and repolarization stages. Duration of the action potential and mAHP in OCM MNs also diminishes half in time between the newborn and the adult ages, but these changes take place gradually, and more slowly than in the GG MNs, between the first two weeks (duration of the action potential) and the three weeks (mAHP) of development. One possible mechanism for the changes observed in action potential width is the increase in channel density or alternatively a more synchronized opening of voltage-gated Na^{+} channels and delayed rectifier K^{+} channels underlying action potentials[117-121]. Both mechanisms are proposed in Figure 5E to explain postnatal alterations in action potentials with age. The increase in channel density[117] may also be a consequence of the previously described changes in the axon initial segment length[116] with development. Considering that firing rate mainly depends on mAHP duration[122,123], a comparison of the mAHP data between the two pools is appropriate since it would produce a higher discharge rate in the adult cells. In Figure 5E, we also propose that a decrement in voltage-activated Ca^{2+} conductance with age, underlying the action potential afterdepolarization, could also be responsible of the decay in mAHP duration with development. This stage is known to depend on a Ca^{2+}-dependent K^{+} current[88,124]. A similar interpretation may be proposed to understand the behavior of other brainstem and spinal MNs[83-85]. The shortening of the mAHP is more evident in OCM (approximately 100%) than in GG MNs (approximately 25%) (Figure 5D)[4,10] as a consequence of a longer time

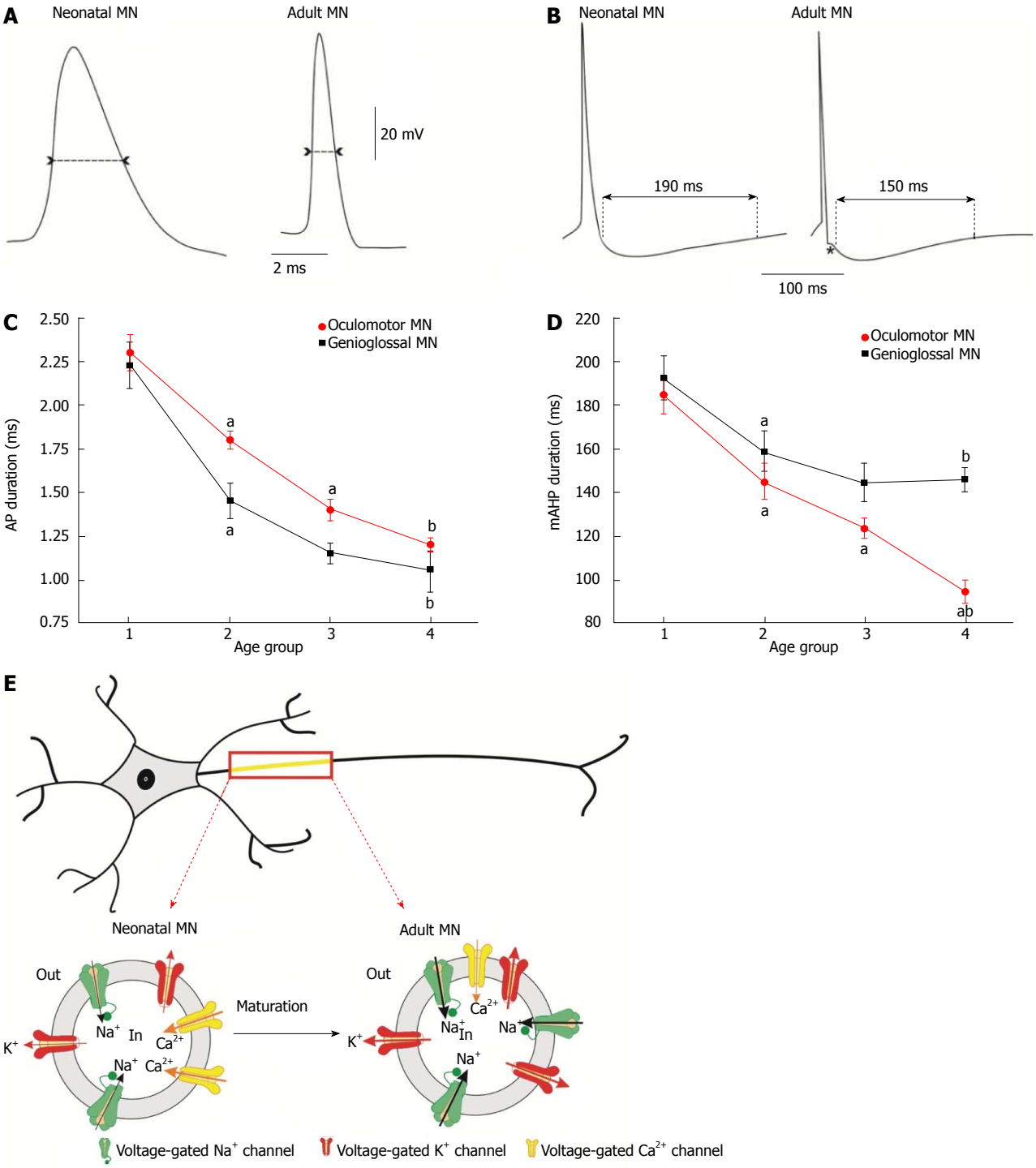

Figure 5 Postnatal maturation of action potential characteristics of motoneurons from genioglossal and oculomotor nuclei. A and B: Recordings illustrating a representative action potential from one neonatal and one adult MN, at two different time scales, emphasizing the duration of the action potential (A) and the duration of the medium afterhyperpolarization phase of the action potential (B). Note the shortening in both phases during development. Also remarkable the presence of afterdepolarization phase in the adult MN (see asterisk); C and D: Plots illustrating the changes on action potential duration (C), and medium afterhyperpolarization phase duration (D) during postnatal development for GG and OCM MNs. Both populations show a decrease in these parameters. However it should be noted that, in GG MNs, changes in the afterhyperpolarization phase cease to decrease at the second postnatal week and, in the OCM MNs, this decrease is bigger and continuous up to P30; E: Schematic drawing illustrating proposed differences in ion channels (number and kinetics) underlying action potentials between neonatal and adult MNs. Note, for the adult MN, a higher number of potassium and sodium voltage gated channels and larger conductances (thick arrows) when compared with the neonatal MN. We also proposed the existence of less voltage gated calcium channels with lower conductances (thin arrows) for the adult MNs. The "a" indicates statistical significance between two consecutive age groups; and the "b" represents statistical significance between age group 1 and age group 4. The age groups 1,2,3 and 4 correspond to P1-P5, P6-P10, P11-P15 and P21-P30, respectively. The data from GG[4] and OCM[10] come from previous studies. MNs: Motoneurons; GG: Genioglossal; OCM: Oculomotor.

window of changes (Figure 5D). Then, the decrement in voltage-activated Ca^{2+} conductance with age must be stronger in OCM MNs, since those conductances are lower in adult OCM than in hypoglossal MNs[125].

TIME-DEPENDENT CHANGES IN FIRING PROPERTIES: FIRING FREQUENCY, GAIN AND MAXIMUM FREQUENCY

At birth, all GG MNs display an adapting discharge pattern (Figure 6A). Later, after the first week, adapting firing pattern is converted to a non-adapting firing (phasic-tonic pattern, Figure 6A). It is possible that the progressive decrease in the duration of the mAHP found in these train discharges is the result of the progressive activation of a Ca^{2+}-mediated K^+ conductance[126] because processes of Ca^{2+} sequestration and extrusion[127] are not well developed in MNs at birth, allowing for an increase in the concentration of intracellular Ca^{2+} with each successive spike. By contrast, and regardless the age group, all OCM MNs repetitively discharge with a phasic-tonic pattern to sustained depolarizing currents. Therefore, the firing pattern is already established at birth in this population[10].

In Figure 6B, firing frequency gain was obtained from the slope of the F/I plot in four representative neurons (two of each MN pool). It is evident from the figure that gains are higher in adult than in newborn MNs in both nuclei (Figure 6C)[4,10]. GG MNs share with OCM MNs[4,10] and spinal MNs[83] a tendency to increase the firing rate with postnatal development. The main difference between the two populations is that the increase in gain extends to P15 in GG MNs but continues up to P30 in OCM MNs, resulting in a higher discharge rate in adult OCM MNs when compared with adult GG MNs (Figure 6C). The balance between tonic inward currents and the outward currents has been suggested to be a major determinant of the F-I relationship[27,91,125,128-131]. Physiological mechanisms that may underlie the trend toward higher discharge frequencies during postnatal development include an increase in the hyperpolarizing-activated mixed-cationic currents[90]; a rise in both persistent sodium conductance and long-lasting calcium current[88,107,109,111]; a decrease in the low-voltage-activated calcium currents[129]; and a reduction of A-type potassium current[84]. Neurotransmitter and trophic factors may be controlling most of these conductances and those underlying the mAHP[23,24,122,132-135].

Figure 6D illustrates changes of maximum frequency with age. Neonatal GG and OCM MNs have lower maximum firing frequency than the one found in the adult. Furthermore, increase in the maximum firing rate takes place between P16-P30 in OCM MNs, whereas in GG MNs the rise continues up to the adult stage[4]. An explanation for a higher frequency during development is a more rapid activation of the delay rectifier or a shorter inactivation stage of the voltage-gated Na^+ channels[136]. Furthermore, the maximum frequency of adult OCM MNs overcomes that of the newborn three times, while the increase in GG discharge is less than twice (Figure 3B). Then, we suggest that extraocular MNs develop a higher pattern of discharge to achieve their function of producing faster contraction times of the extraocular muscles when compared with the genioglossus and other skeletal muscles[137,138].

TIME WINDOWS OF CHANGES IN MEMBRANE PROPERTIES

A time window exists when a brain circuit that subserves a given function is specifically receptive to acquiring certain kinds of information, or when the circuit needs a signal for their normal development[28]. Changes in morphological and electrophysiological properties of the membrane can, for simplification purposes, be distributed in 3 distinct time windows, *i.e.*, the first, second and third-fourth weeks of life (Figure 7). We first describe changes in GG MNs (left of the Figure 7) and then changes in OCM MNs (right of the Figure 7).

In GG MNs the dendritic tree is simplified and gap junctions disappear during the first postnatal week, while the membrane surface is maintained. The action potential and the hyperpolarization stage diminish. The firing pattern becomes phasic-tonic. During the second week, the dendritic tree completes its simplification by augmenting its dendritic length. Also, membrane resistance and time constant decrease and there exists a compensatory change in the rheobase. During the third postnatal week, the dendritic complexity increases until reaching values found at birth. This increase was produced by the formation of new dendritic branches, as a consequence of the enlarging diameter of primary dendrites and the total dendritic surface. This dendritic re-organization was elaborated, in an asymmetrical way, on some axes mainly (*i.e.*, the ventrolateral axis) and it produces an increase of the dendritic length. During this third week, firing frequency gain and maximum frequency reach a significant increase that had slowly started at birth.

For the OCM MNs, the first postnatal week is characterized by an increase of the dendritic surface which shows larger dendritic complexity. At the same time, we can observe changes in the membrane passive properties (input resistance and time constant). During the second week, this dendritic complexity gets simplified to values found at birth, the dendritic length increases by the use of the pre-existent membrane, and the action potential diminishes in duration. The simplification and elongation of the dendritic trees is completed during the third postnatal week, and dendrites grow in length, preferably along some spatial axes in order to achieve the dendritic spatial pattern typical of the adult population. Also during the third week, the membrane active properties change: (1) the decrease in duration of mAHP and rheobase (due to a drop in the voltage threshold) are completed; (2) the increase of firing frequency gain started at birth is concluded; and (3) there is an increase of the maximum frequency.

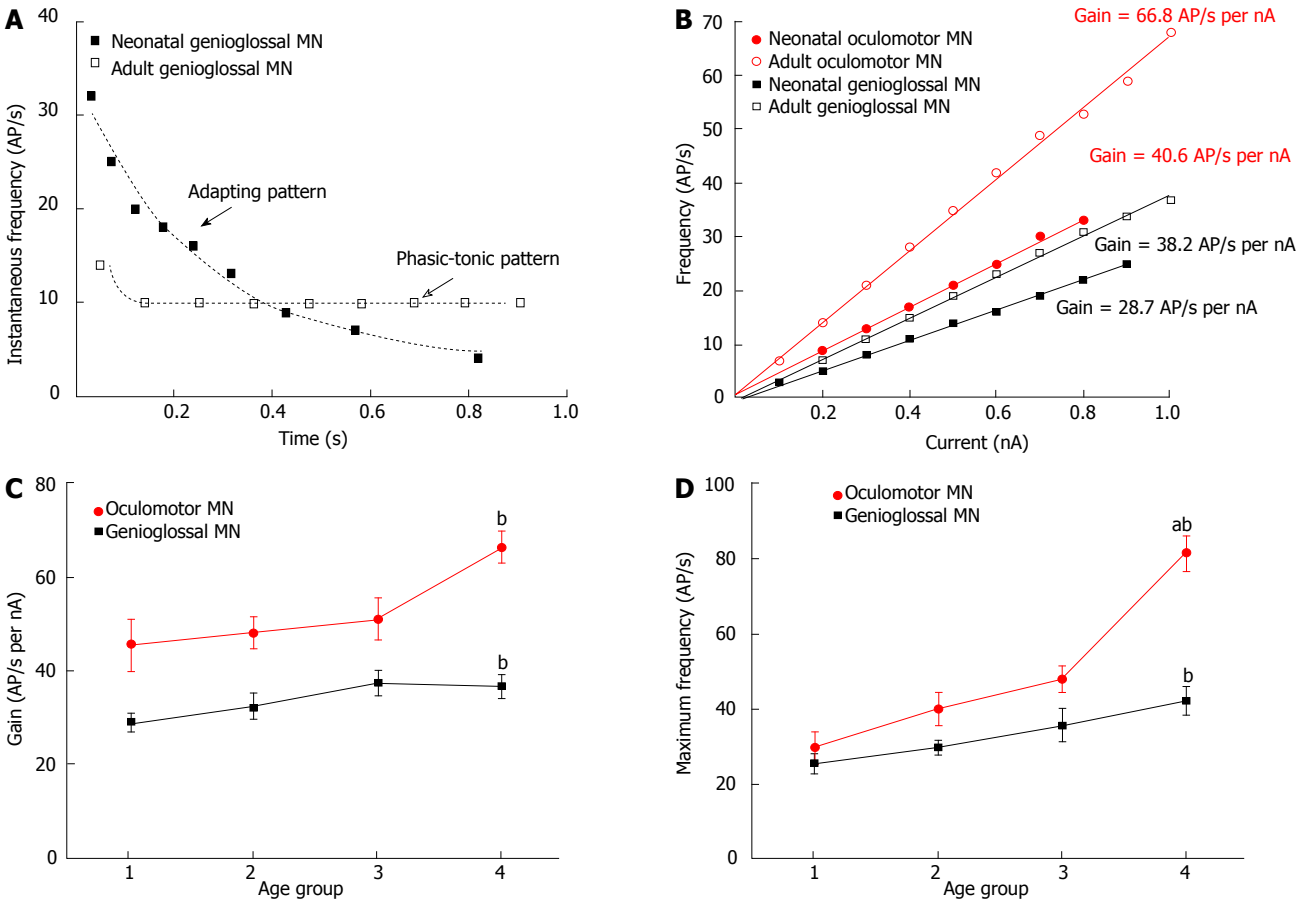

Figure 6 Postnatal maturation of repetitive firing properties of motoneurons from genioglossal and oculomotor nuclei. A: Instantaneous firing frequency evoked by depolarizing current stimulus of 0.3 nA for a representative MN from the GG nucleus. Only the neonatal MN shows an adapting firing pattern while the adult MN shows a phasic-tonic pattern; B: Relationship between current (I) and firing (F) for a representative neonatal MN (filled red circle) and an adult OCM MN (open red circle); and for a representative neonatal MN (filled black square) and an adult MN (open black square). The slope of the I-F relationship is the gain; C, D: Plots illustrating the changes on gain (C) and maximum firing frequency (D) during postnatal development for GG and OCM MNs. Note that gain and maximun firing frequency increase with age, although these increments are larger for OCM MNs. The "a" indicates statistical significance between two consecutive age groups; and the "b" represents statistical significance between age group 1 and age group 4. The age groups 1,2,3 and 4 correspond to P1-P5, P6-P10, P11-P15 and P21-P30, respectively. The data from GG[4] and OCM[10] come from previous studies. MNs: Motoneurons; GG: Genioglossal; OCM: Oculomotor.

TIME WINDOWS IN THE CONTEXT OF DEVELOPMENT OF THE RESPIRATORY AND OCM SYSTEMS

Time windows for changes in membrane properties of MNs are probably determined by extrinsic signals (synaptic inputs and growth factors) related to the circuits in which they participate. The brain-derived neurotrophic factor, when acting through its high-affinity receptor TrkB, has intensively been studied in brainstem neurons during development because of its growth-promoting and trophic effects, including those involved in respiratory control and normal breathing[30,139,140]. It it known that the loss of specific trophic signaling modifies the development of different subpopulations of motoneurons in heterogeneous way[32]. Thus, the lack of cardiotrophin-1[141,142] or IGF-1 significantly reduces the number of brainstem motoneurons[143]. GG and OCM MNs are brainstem motoneurons that innervate tongue and extraocular muscles, respectively. One of the main functional differences between the two muscles lies in the fact that the tongue is present in various motor tasks, including suckling, swallowing and respiration necessary for the animal from the moment they are born, while the extraocular muscles should be ready to work at P21. For example, breathing, which is genetically determined to work at birth, is the result of a well-defined developmental program[144]. This difference in muscle functions could explain the distinct temporal sequences of changes between the two populations of motoneurons studied here. The shortening in action potential duration and mAHP occurs in GG MNs during the first week[4], while the same appears at P15-P20 in OCM MNs[10]. Furthermore, the maximum firing discharge is reached after the third postnatal week in both populations but its increase is much more localized for OCM MNs in the third week[4,10]. Although the evidence is not clear, we could assume that the earliest shortening of the action potential and mAHP in GG MNs correlates with tongue functions that become mature just after birth. The slower frequency of discharge found in newborn MNs is appropriately matched to the slower contraction times found in neonatal skeletal muscles[4,10,145]. The matching of MN

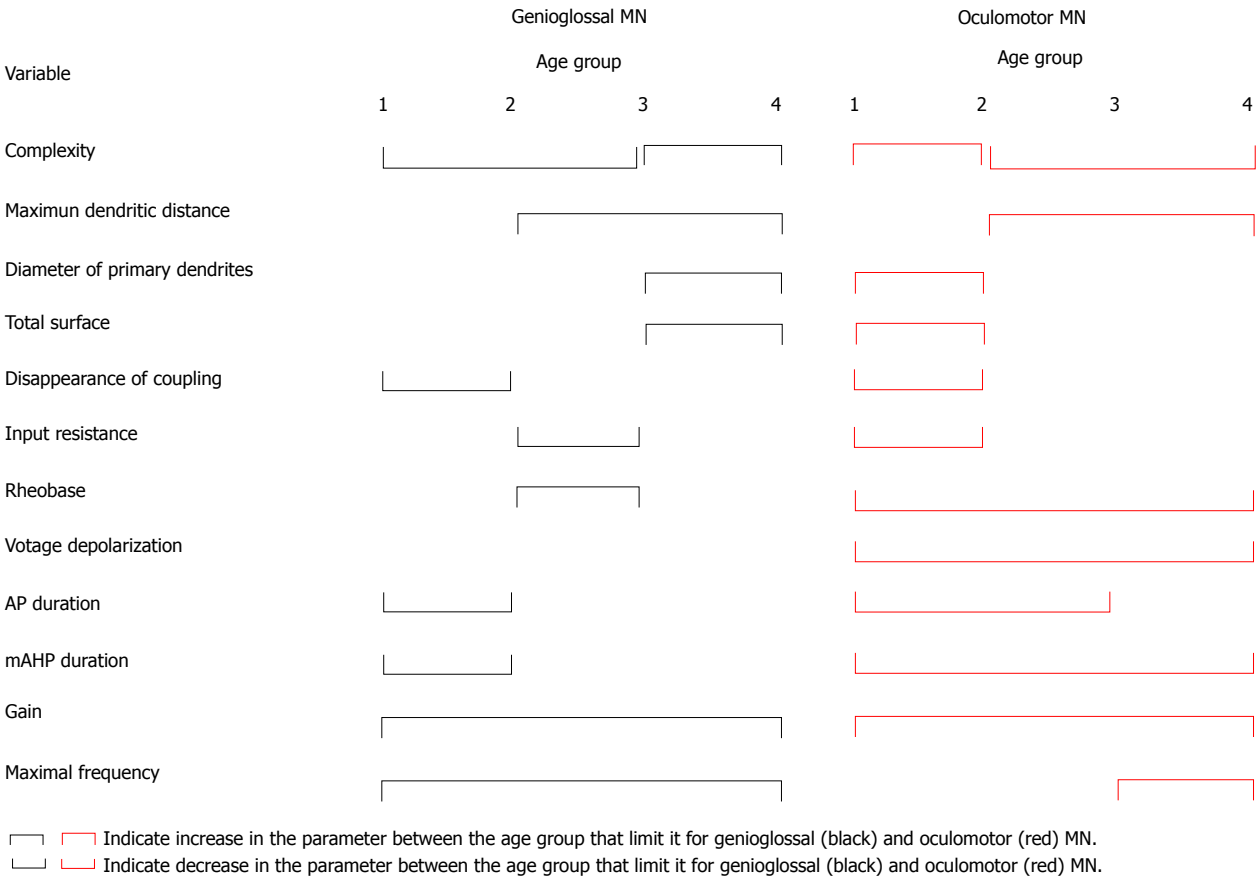

Figure 7 Summary of changes during postnatal periods for genioglossal (black) and oculomotor (red) motoneuron. MNs: Motoneurons.

and muscle properties during postnatal development has been previously described[146,147]. As the speed of respiratory muscle contraction increases with age, the adapting firing pattern is converted to a non-adapting one. This conversion to a faster, non-adapting firing (phasic-tonic pattern) may be required to sustain a fused tetanus of the muscular contraction. Modifications in the maximum firing rate in GG MNs late in development may enable more refined motor functions, and also allow for other tasks including mastication, sneezing, coughing, emesis and "vocalization"[1,2,4,6,148].

The changes described in the reshaping of the dendritic tree and passive membrane properties of GG motoneurons must be understood in the context of the development of respiration in the rat in the first 3-4 weeks of life[4,6]. Respiratory frequencies are characterized by a constant increase with age that reaches peak values at P13 and declines onwards until P21. During the first postnatal week, the absolute tidal volume adopts relative plateau shape, followed by a constant rise until P21[149] denoting the achievement of more mature, deeper, and slower breaths. Furthermore, the second week after birth shows a highly plastic and narrow window of respiratory maturation. This time window is a period in which the neuronal circuits that subserve respiratory control are structurally and/or functionally shaped[150]. These time windows in GG MNs coincide with the decrease in resistance[4], and also with a critical period in the rat (around P12-13) when a functional transient imbalance between excitation and inhibition is found. This imbalance is characterized by a decrease in the amplitude and frequency of excitatory postsynaptic current and an increase in the amplitude and frequency of inhibitory postsynaptic currents[63] as a result of a transient reduced expression of brain-derived neurotrophic factor and TrKB expression[30]. Concurrently with the abrupt fall in brain-derived neurotrophic factor at P12-13, the expression of excitatory neurochemicals (glutamate and NMDA receptor subunit 1) is drastically reduced, whereas that of inhibitory neurochemicals ($GABA_A$, $GABA_B$ receptors and glycine receptors) is significantly enhanced in hypoglossal MNs and in other respiratory-related nuclei[149]. Then, a proliferation of excitatory synaptic inputs must take place later on in order to compensate for the decrease in input resistance and the decrease of excitation to reach threshold. That proliferation of synaptic inputs may be coincident with the increase in surface area that GG MNs exhibit during the third postnatal week[5]. In addition, the lowest values in rheobase are found in GG nuclei at birth[4], and have been understood to ensure the recruitment of most MNs so that suckling movements can be executed, a critical motor task after birth[4,85]. Also for GG MNs, we propose that voltage-gated K^+ channels undergo an increase in number and kinetics during development, and they may be critical determining firing frequency and maximum frequency. The modulation of

these K^+ channels causes genioglossus inhibition due to postsynaptic inhibition of GG MNs in rapid eye movement sleep[151,152], which in turn produces periods of upper airway motor suppression, atonia of the GG muscle, hypoventilation and obstructive apneas. Patency of the upper airway (*i.e.*, tone of the genioglossus muscle) is essential to maintain ventilatory processes during wakefulness as well as nonrapid eye movement (NREM) and rapid eye movement (REM) sleep[152]. Then, defects in maturation patterns in GG MNs may contribute to the development of sleep apnea and other cranial motor disorders including Rett syndrome, and sudden infant death syndrome[149,153].

OCM MNs drive eye movements following vestibular and visual sensory signals[154]. MN excitability, synaptic circuitry and extraocular muscles mature together. However, to establish that the maturation of OCM MNs is driven by a change in both their afferents and extraocular muscles is still to be proved. We may, however, accept that the early developmental processes go along in OCM MNs, vestibular and visual signals and the extraocular muscles they innervate. For example, rodent extraocular muscles are very immature when they are born[155] and their muscle fibers contain supernumerary motor nerve terminals[156]. Maturation in rats has been proven to be ready one week after birth in all vestibular components: the vestibular organ, hair cell sensitivity, and the circuitry that transmits the signal to the vestibular nucleus[157]. Likewise, the circuitry that transmits the signal from the vestibular nuclei to the ocular MNs is ready before P10[157-159]. On the other hand, visual deprivation or lesion to hair cells cause maldevelopment of the extraocular muscles[160,161] and impairment in the vestibulo-ocular reflex[162]. Should visual sensory signals participate in the development of OCM MNs, it would have to be after P12, when the eyelids open. In addition, rodents present eye movements that are evoked by visual and vestibular stimuli from about P21 onwards, although the precision of these reflexes augments later on to achieve clear vision during self-motion[70,71]. Therefore, the maturation of distinct pathways that drive optokinetic and vestibulo-ocular reflexes, including the cerebellar-dependent mechanisms, is ready in the first 3-4 weeks after birth[70]. OCM MNs grow and lower their input resistance with age[9-11,13]. We have found that passive membrane properties mature shortly after birth (P1-P5), while changes in active properties require a longer time scale[10]. Similar findings have been described for vestibular neurons[163] with the conclusion that changes in membrane properties with development happen to enable mature firing properties when required by the proper optokinetic response. The same may apply to the OCM MNs. Eyelids open at about P12 and eye movements evoked by visual and vestibular stimuli occur after the third week after birth[70], when MNs finally present adult firing properties[10,11]. Visual synaptic inputs to MNs may determine recruitment threshold[12,21]. In accordance with this last finding, the decrease in rheobase with age in OCM MNs would guarantee the recruitment of most of these cells after P21, in order to lead to eye movements[9]. With postnatal development, the most active MNs have competitive advantages in muscle synaptic refinement[156]. Extraocular MNs generate burst-tonic activity that enables rapid shifts (saccades) and fixation of the eye orbit[17-19]. Furthermore, muscle derived factors (neurotrophins) are important to ensure neuronal survival during maturation and their range of actions support the phasic and tonic activities of MNs in conducting eye movement[164]. We conclude that lower rheobase and higher maximum frequency of OCM MNs would be needed in the third week of development to generate faster contraction times, shifts (saccades) and fixation of the orbit of the eye[137,138].

CONCLUSION

From our data on GG and OCM MNs[1-13] we conclude that there exists a clear-cut developmental program that produces age-related changes. Common to both populations are modifications in dendritic structure (complexity, length and size), passive properties (input resistance, time constant, and rheobase) and active properties of action potential and firing pattern. However, the time windows of changes in these properties are different and the sequences are even inverted between GG and OCM MNs. Then, most of the described temporal windows of the changes in membrane properties could be understood to be related with the maturation of the respiratory and OCM systems. However, future research in the field would need to address the following issues: (1) how (genetically determined) intrinsic factors shape dendritic branching structure and membrane properties; (2) what processes may be under the control of the targeted muscles, such as motoneuronal survival and electrotonic coupling; (3) how postnatal development of the respiratory and the vestibulo-OCM circuitries determines the dendritic enlargement of dendrites to reach adult territories, as well as changes in passive membrane properties of GG and OCM MNs, respectively; and (4) how active membrane properties of GG and OCM MNs rely on the activation of circuits at the onset of breathing and eye opening.

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