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EDITORIAL

New findings showing how DNA methylation influences diseases

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Abstract

In 1975, Holliday and Pugh as well as Riggs independently hypothesized that DNA methylation in eukaryotes could act as a hereditary regulation mechanism that influences gene expression and cell differentiation. Interest in the study of epigenetic processes has been inspired by their reversibility as well as their potentially preventable or treatable consequences. Recently, we have begun to understand that the features of DNA methylation are not the same for all cells. Major differences have been found between differentiated cells and stem cells. Methylation influences various pathologies, and it is very important to improve the understanding of the pathogenic mechanisms. Epigenetic modifications may take place throughout life and have been related to cancer, brain aging, memory disturbances, changes in synaptic plasticity, and neurodegenerative diseases, such as Parkinson's disease and Huntington's disease. DNA methylation also has a very important role in tumor biology. Many oncogenes are activated by mutations in carcinogenesis. However, many genes with tumor-suppressor functions are "silenced" by the methylation of CpG sites in some of their regions. Moreover, the role of epigenetic alterations has been demonstrated in neurological diseases. In neuronal precursors, many genes associated with development and differentiation are silenced by CpG methylation. In addition, recent studies show that DNA methylation can also influence diseases that do not appear to be related to the environment, such as IgA nephropathy, thus affecting the expression of some genes involved in the T-cell receptor signaling. In conclusion, DNA methylation provides a whole series of fundamental information for the cell to regulate gene expression, including how and when the genes are read, and it does not depend on the DNA sequence.

Key words: DNA methylation; Stem cells; Enhancer; IgA nephropathy; Gene regulation

Core tip: DNA methylation in eukaryotes acts as a hereditary regulation mechanism that influences gene expression and cell differentiation. Recently, we have begun to understand that the features of DNA methylation are not the same for all the cells. Major differences have been found between differentiated cells and stem cells. However, epigenetic modifications may take place throughout life and influence various diseases, and they are very important for improving the understanding of pathogenic mechanisms. New studies show that DNA methylation can also influence diseases that do not appear to be related to the environment.

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INTRODUCTION

In 1975, Holliday and Pugh as well as Riggs independently hypothesized for the first time that DNA methylation in eukaryotes could act as a hereditary regulation mechanism to influence gene expression and cell differentiation. Epigenetics was born as the study of inheritable changes in the genome that occur without modification of the DNA sequence and affect its functionality. DNA methylation is an extremely important mechanism. Together with covalent modifications of histones (proteins that compact the DNA), methylation modifies the chromatin structure and the accessibility of DNA to the regulation factors of gene expression.

DNA methylation density strongly varies with each chromosome. The sub-telomeric regions near the extremities often show a high methylation rate, which is important for controlling telomere length and recombination. In general, a lower methylation density is observed at the level of "CpG islands" and transcription initiation sites[1]. In 2008, a definition of epigenetic characteristics was formulated during the Cold Spring Harbor Meeting as a "stably inheritable phenotype deriving from a chromosomal alteration not connected to DNA variations"[2].

DNA methylation is maintained throughout the life of the affected cells and can be transmitted to subsequent cellular generations without modification of the DNA sequence. The processes responsible for epigenetic changes can take place both before and after transcription. In the first case, they are mainly represented by DNA methylation, which is bound by a covalent bond (and therefore reversible) to a methyl group coming from the universal methyl donor S-adenosylmethionine in position 5 of the cytosine residue of the CpG dinucleotide (Cytosine-phosphate-Guanidine). The phenomenon is due to the intervention of the specific enzyme DNA methyltransferase (DNMT).

The hypermethylation of DNA determines the "silencing" of the gene of interest, while hypomethylation causes activation[3]. Anomalous chromatin states that lead to abnormal gene expression patterns have been defined as epimutations, which have been detected in numerous diseases, including cancer. Epimutations can affect one or both alleles of a gene. Epimutations in cancer usually occur in somatic cells and cause cancer progression[4-8].

FEATURES OF DNA METHYLATION ARE NOT THE SAME FOR ALL CELLS

Recently, we have begun to understand that the features of DNA methylation are not the same for all cells. Major differences have been found between differentiated cells and stem cells. For example, 99.98% of the methylated DNA regions in normal cells, such as fibroblasts, are rich in C and G, whereas the methylation in stem cells is concentrated in sequences that are rich in A and T. Moreover, embryonic cells that are induced to differentiate lose the methylation at the level of non-CG sequences, while they maintain the methylation in the sequences rich in C and G. This indicates that widespread methylation at the non-CG level is lost during differentiation.

Previous studies have hypothesized the existence of methylation almost exclusively in C and G-rich sequences in mammals, but observations led to the supposition that

non-CG methylation is a general feature, at least for human embryonic stem cells. The absence of non-CG methylation in fibroblasts coincides with a reduced presence of *de novo* DNMTs (DNMT3A, DNMT3B, and DNMT3L)-that is, enzymes that catalyze the addition of methyl groups in previously unmethylated residues. In contrast, "maintenance" methyltransferases reproduce the methylation pattern in a DNA strand based on what is present in the other filament[1].

A positive correlation between gene expression and methylation density is also observed at the level of non-CG sequences. The most expressed genes contain three times higher methylation density than unexpressed genes. However, no correlation has been detected between CpG methylation density and gene expression in stem cells. A particularly high methylation density has been observed for genes involved in RNA processes, such as splicing and RNA metabolic processes. Unexpectedly, an enrichment of non-CG methylation was found at the level of the antisense strand of the coding regions of genes, but the potential roles of this methylation are currently unknown.

Numerous studies have documented a correlation between DNA methylation and the ability of some proteins to interact with their target sequences. A decrease in DNA methylation density has been noted in correspondence with the protein interaction sites.

Another very important modality of gene expression regulation involves the "enhancer" regions, which are short DNA sequences that can to bind activating proteins, which in turn facilitate the recruitment of RNA polymerase and thus the transcription of the regulated gene. A decrease in methylation has been observed at the level of enhancers specific for fibroblasts. Conversely, at the level of specific stem enhancers, the methylation density does not change in either the embryonic stem cells themselves or in fibroblasts. This indicates the maintenance of these elements in an unmethylated state, thus preventing interference in the protein-DNA interaction process. The specific type of de-methylation (non-CG in stem cells and CG in fibroblasts) could indicate the use of different types of methylation specific to each cell type. Another paradigm of DNA methylation is that it controls aspects of cell differentiation. Obviously, this implies that methylation patterns vary in different cell types, as documented in several studies[9,10].

METHYLATION INFLUENCES VARIOUS PATHOLOGIES

Interest in the study of epigenetic processes has been inspired by their reversibility and their potentially preventable or treatable consequences[11,12]. It is easy to understand how methylation influences various pathologies and the importance that it covers to understand better pathogenic mechanisms. In recent years, several studies have shown that DNA methylation influences many diseases. Cancer has been the most studied among the numerous diseases in which epigenetic modifications are the object of greater attention. DNA methylation also has a very important role in tumor biology.

Cancer is considered an essentially genetic disease, in which mutations alter the functioning of genes, causing the cell to proliferate in an uncontrolled manner. In recent decades, however, numerous indications have led to the suspicion that epigenetic factors-particularly DNA methylation-may be involved in the genesis of a tumor. In carcinogenesis, many oncogenes are activated by mutations[13-16]. However, many genes with tumor suppressor function are "silenced" by the methylation of CpG sites present in some regions[17].

We have known that epigenetic changes are associated with cancer, but until a few years ago, we did not know if they were the cause or a consequence of the disease. Recently, the development the new "epigenetic engineering" approach in some studies has allowed us to verify that even changes in DNA methylation alone can induce cancer. In fact, in new research, Yu *et al*[18] created a line of genetically modified mice where a small fragment of DNA adjacent to the p16 gene behaved like a magnet that attracted DNA methyltransferases and methyl groups, which hypermethylated the gene promoter and blocked the possibility of transcription. p16 has the function of blocking the cell cycle and preventing mitosis when necessary. For this reason, the p16 gene is considered a tumor-suppressor gene.

The results of the study showed that in the population of transgenic adult mice, the gene encoding p16 is more substantially activated during aging. The incidence of spontaneous tumors was higher than that in the control population of normal mice, in which the tumor suppressor gene continued to act regularly. Obviously, this kind of regulation can be extended to several other oncogenes in several other diseases. This result has profound implications for future studies because epigenetic changes are

potentially reversible. Therefore, new epigenetic therapies may be very effective for both tumors and other diseases, such as neurodevelopmental diseases, obesity, and diabetes.

The role of epigenetic alterations has also been demonstrated in neurological diseases. In neuronal precursors, many genes associated with development and differentiations are silenced by CpG methylation. The regulation of the proteins that bind to the methylated CpG is subject to mutations, duplications, and insertions. One example of a condition that depends on these processes is Rett's syndrome, which involves severe mental retardation linked to the X chromosome. Studies carried out with animal models of this disease report very interesting results in that the modifications of the CpG are at least partially reversible[19]. Epigenetic modifications may take place throughout life and have been related to brain aging, memory disturbances, and changes in synaptic plasticity[20]. The resulting alterations are increased over the years[21] and become significant in various neurodegenerative diseases, such as Parkinson's disease and Huntington's disease[14,22].

Various observations have led to the suspicion of the existence of epigenetic mechanisms in the pathogenesis of asthma, which is present in both twin homozygotes in as much as half of the cases[23]. There are complex interactions between genetics and the environment, which could lead to epigenetic modifications of the genome. One example is the demonstrated interaction between maternal smoking during pregnancy and the activity of the interleukin-1 receptor antagonist in newborns, which is associated with a significant increase in the risk of asthma[24]. In contrast, exposure to some endotoxins *in utero* appears to have a protective effect[25]. Moreover, many studies on the genetics of asthma have shown the existence of gene de-regulations that can be explained with only epigenetic alterations and DNA hypomethylation of 14 CpG sites that are gained after birth and linked with childhood asthma[26,27].

Recent studies show that DNA methylation can also influence diseases that do not appear to be related to the environment, such as IgA nephropathy (IgAN). This condition is the most common form of primary glomerulonephritis worldwide and has a strong genetic component. DNA methylation in the CD4+ T cells of IgAN patients influences the expression of some genes involved in the T-cell receptor signaling, which is the pathway that transfers the signal for the presence of antigens and activates the T-cells[28]. In particular, TRIM27 and DUSP3 genes were found to be hypomethylated in correspondence to the site that modulates their transcription, and these genes are upregulated in the CD4 T cells of IgAN patients.

The DNA region encoding vault RNA 2-1 (VTRNA2-1) non-coding RNA was also found to be hypermethylated, leading to its down-regulation. In turn, following CD3/CD28 T-cell receptor (TCR) stimulation, the lower levels of VTRNA2-1 cause a decrease in the proliferation of CD4+ T-cells, which plausibly occurs through the activation of the interferon-inducible kinase protein kinase R. Lower VTRNA2-1 levels also increase transforming growth factor beta expression. Together with DUSP3 and TRIM27, the increased transforming growth factor beta expression impairs the proliferation and activation of CD4+ T-cells, thus reducing the effect of the CD3/CD28 activation[28]. This deregulation causes reduced TCR strength and a T-cell anergy-like status. The lower activation of CD4+ T-cells and the lower TCR strength can determine Th1 polarization with higher interleukin 2 production in some biological settings. The aberrantly methylated DNA regions of CD4+ T-cells in IgAN patients thus offers a way to improve the understanding of the molecular mechanisms implicated in this disease. They could also lead to a new point of view for new therapeutic targets for the treatment of the IgAN.

In addition to these DNA methylation processes, we should also consider that various other inheritable mechanisms of post-transcriptional gene regulation can be used by cells, particularly the synthesis of non-coding microRNA, which binds to the corresponding messenger RNA and causes degradation or inhibition[29]. Recent studies also reveal an interesting interaction between these two kinds of epigenetic regulating systems. Gene expression can be affected by DNA methylation operating at a distance through the methylation or demethylation of the regulatory regions of miRNAs. The diversity of miRNA targets may produce the concurrent regulation of numerous biological pathways, such as apoptosis, cell proliferation, and migration.

Many *in vitro* and *in vivo* studies have shown that even epigenetic modifications of microRNAs can intervene in the pathogenesis of atherosclerotic lesions. For example, miR-33 inhibits the genes involved in the ability to expel cell cholesterol, the metabolism of high-density lipoproteins, lipid oxidation, and glucose metabolism. In mice, miR-33 deficiency is associated with a reduction of induced atherosclerotic lesions[30]. In neuroblastoma, a cluster of aberrantly methylated miRNA genes could lead to impaired regulation of the cell cycle, apoptosis, and the control of V-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog expression[31].

These data show that a complex arrangement of connections between epigenetically managed miRNAs and target genes may affect the control of cell homeostasis at different levels.

CONCLUSION

The knowledge is changing in regard to the regulation of pathways and biological processes in living organisms, including humans. DNA methylation provides a whole series of fundamental information for cells to regulate their gene expression, including how and when the genes are read, and it does not depend on the DNA sequence. As discussed, these reversible DNA modifications are influenced by the surrounding context and can heavily influence diseases.

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MINIREVIEWS

Autism and carnitine: A possible link

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Abstract

Patients with autism spectrum disorders (ASD) present deficits in social interactions and communication, they also show limited and stereotypical patterns of behaviors and interests. The pathophysiological bases of ASD have not been defined yet. Many factors seem to be involved in the onset of this disorder. These include genetic and environmental factors, but autism is not linked to a single origin, only. Autism onset can be connected with various factors such as metabolic disorders: including carnitine deficiency. Carnitine is a derivative of two amino acid lysine and methionine. Carnitine is a cofactor for a large family of enzymes: the carnitine acyltransferases. Through their action these enzymes (and L-carnitine) are involved in energy production and metabolic homeostasis. Some people with autism (less than 20%) seem to have L-carnitine metabolism disorders and for these patients, a dietary supplementation with L-carnitine is beneficial. This review summarizes the available information on this topic.

Key words: Autism; Carnitine; Neurodevelopmental; Metabolism; Pathophysiological bases

Core tip: Autism spectrum disorder is characterized by impaired communication, altered social skills, stereotypical behaviors and limited interests. The pathophysiological bases of autism have not been defined yet. Several publications have pointed a possible connection between autism and carnitine deficiency. Carnitine is a cofactor for a large family of enzymes: the carnitine acyl transferases. Through their action these enzymes are involved in energy production and metabolic homeostasis. Low plasma carnitine were reported in autism patients and for some of them, defects in L-carnitine metabolism have been reported. This review summarizes the available information on the possible link between autism and carnitine.

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AUTISM

Description

Autism is a heterogeneous neurodevelopmental disorder actually known as Autism spectrum disorders (ASD). It is characterized by a dyad of symptoms: impaired communication and altered social skills[1]. ASD symptoms include (1) persistent deficits in initiating and sustaining social interaction and social communication; and (2) limited, repetitive and stereotypical patterns of behaviors and interests.

The impact of ASD on people can be very dissimilar: some people with ASD can live independently, others require life-long care and support. The onset of this disease occurs, typically, in early childhood usually before the age of three. For some patients, the symptoms will become apparent later when daily autonomy requirements will not be handled by the patient. According to the Diagnostic and Statistical Manual of mental disorders (DSM-5®), the symptoms must appear during the childhood to be considered as autism[1].

Most people affected by autism are healthy otherwise but for some of them, autism is associated with other health problems. Among those pathologies associated with autism, one can find metabolic disorders such as phenylketonuria, chromosomal abnormalities, infectious diseases such as rubella or neurocutaneous disorders.

In most cases, deficits are severe enough to affect the personal life: the different aspects of social, educational, occupational life are generally affected in people affected by ASD. The spectrum is large and among patients, a full range of mental abilities and communication skills can be observed[2].

In the literature, some theories explain autism by cognitive deficit. Baron-Cohen studied the hypothesis of a deficit of theory of mind and showed that patients with ASD have difficulties in imputing mental states (beliefs, desires, intentions, emotions, *etc*.) to others[3]. It has been proposed that people with ASD have a deficit of executive functions. Evaluations of cognitive functions objectivize deficits in inhibition, cognitive flexibility and working memory. Happé and Frith[4] developed another theory indicating that a person with ASD has a weak central coherence; this theory suggested that a person with autism tends to be more focused on details: the perception of a person with ASD can be defined as fragmented. There are other theories that have been developed to explain what happens in ASD patients: like the perception and sensory theory[5].

Evolution

When ASD is diagnosed in childhood, the symptoms tend to persist during adolescence and adulthood.

With appropriate interventions, the autistic spectrum can be improved: behavioral treatment can improve communication and social behavior, usually associated with a positive impact on wellbeing for people with ASD and their family.

Data and statistics

In the 60th, the prevalence of ASD was estimated to be around 4-5 in 10000, today this number is around 100 (or even more) in 10000 people[6,7] and some authors, even reported a prevalence of 3.6%[7]. This increase may not find its origin in genetic and thus, environmental factors may play a role in the onset of ASD[8]. This rise may also be due to a more efficient diagnosis and a better detection of the disease. For ASD, a male-to female ratio of 3.75:1 has been found[9].

ASD can be diagnosed as early as 2 years old, but most children are not diagnosed with ASD until the age of 4. Usually, the age of diagnosis depends of the severity of ASD. The DSM-5 defined 3 severity levels which depend on the requiring support. A schematic representation of the major features found in ASD patients is summarized in Figure 1.

Parents who have a child with ASD have a 2%-18% risk of having a second child who is also affected[10]. This data can be linked to the genetic aspects of ASD.

ASD is found in every country and in every ethnic group and in both sexes. Reports indicate that prevalence might be different according to the ethnical origin[11] even if

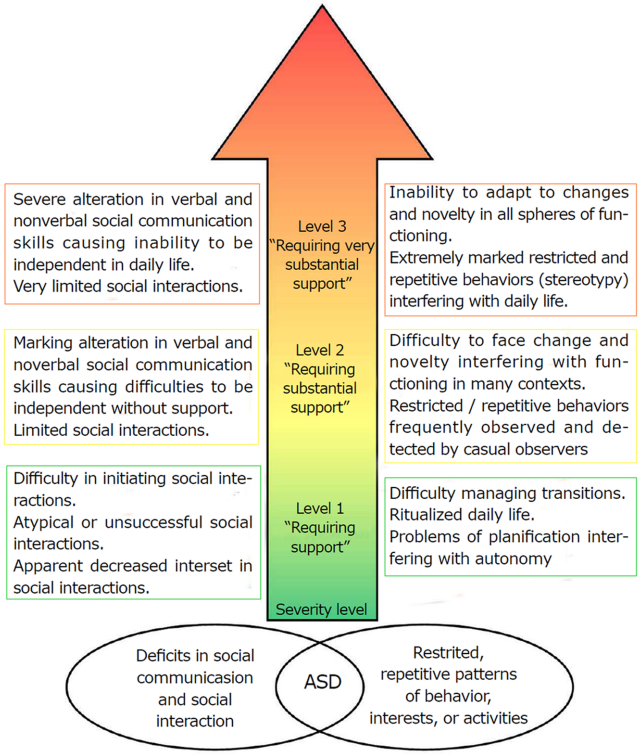

Figure 1 Schematic representation of the major symptoms observed in autism spectrum disorders according to the degree of severity of the disease. ASD: Autism spectrum disorders.

contradictory data suggest that more standardized protocol for diagnosing are required. The differences among these numbers in various countries may also be due to the lack of homogeneity that exists all around the world for diagnosing autism. Professionals agreed to homogenize diagnostic criteria. The main diagnostic guides *i.e.*, the DSM-5 edited by the American Psychological Association (APA) and the International Classification of Diseases 11th Revision (ICD-11) published by the World Health Organization (WHO) are considering this problem.

For several years, ASD is recognized as a public health problem, some people consider ASD as becoming epidemic and as a target for health policy. The support of people with ASD is costly; in fact, people with ASD need more medical examinations and drug prescriptions than most healthy people. An important proportion of children with ASD requires special educational services and some stay in health institution at adulthood. In 2014, in the United States, the total cost for children with ASD was estimated between 11.5 billion to 60.9 billion United States dollars per year[12].

Pathophysiology - Causes

The pathophysiological origin of ASD has not been defined yet. Many factors have been suggested including genetic and environmental factors. These aspects are extensively detailed in a recent review[13]. What seems clear today is the fact that autism is not linked to a single origin. Autism can be associated with many factors, and among those, metabolic disorders can possibly increase the risk of the development of autism.

Some people with ASD also have other health problems, including anxiety and depression, epilepsy, attention deficit hyperactivity disorder (ADHD). In people with ASD, the intellectual level is extremely variable, ranging from profound impairment to higher levels.

In most cases, the etiology of ASD is not known, but a genetic factor, involving possibly 15 or more loci, is widely has been proposed for contributing to the development of ASD[14]. ASD traits are also found in patients affected by several genetic diseases such as Rett syndrome or Angelman syndrome[15].

The strong heterogeneity among individuals with ASD has limited the pure genetic implication[16].

Among potential environmental factors the role of perinatal factors was studied by Gardener *et al*[17], they performed a meta-analysis of the association between perinatal and neonatal factors and the risk of autism. In this study they described associations

between more than 60 potential perinatal risk factors and ASD[17].

Durkin *et al*[18] showed that the age of the parents is associated with the risk of ASD for the children. They noticed that the risk is even more pronounced for the elder children. Premature birth has been identified as a risk factor[19]. Prematurity (infants born at < 37 wk of gestation) and low birth weight (< 2500 g) have also been examined as a risk factor for the development of ASD, among several other potential risk factors[20]. Recently a few studies have associated ASD and pesticides: pesticide exposures during pregnancy is a risk factor for ASD[21].

Some people with ASD have metabolic disorders and/or health problems such as mitochondrial dysfunction and gastrointestinal abnormalities. More than thirty years ago, Coleman and Blass[22] suggested an abnormality in carbohydrate metabolism in individuals with ASD, several years later, it was proposed that ASD may be a disorder associated with an impairment in mitochondrial function[23]. Recently a meta-analysis examined the possible link between mitochondria and autism, the conclusions of this study are that it is not clear if mitochondrial dysfunction contributes to the development or pathogenesis of ASD or if mitochondrial dysfunction is just an epiphenomenon of ASD[14].

CARNITINE

Carnitine occurs in two racemic forms: L- and D-carnitine. In the human body, only the L-isomer is present. L-carnitine is an amino acid derivative found in almost any cell in the body. When discovered, a century ago, L-carnitine was considered as a vitamin as it was shown that the development of a worm *(Tenebrio Molitor*) was dependent on L-carnitine. Several decades later, it was shown that mammals are able to synthesize L-carnitine and subsequently, L-carnitine was not considered as a vitamin anymore.

L-carnitine is mainly found in muscles where it plays a major role in the use of fatty acid for energy production and carnitine found in the human body can either come from an endogenous synthesis or from the foodstuffs.

L-carnitine biosynthesis

L-carnitine biosynthesis is performed with 2 ultimate precursors: lysine and methionine and the enzymatic reactions involved in this synthesis requires several cofactors: vitamin C, iron, vitamin B6 and niacin (Figure 2).

The first step corresponds to the methylation of lysyl residues included in various proteins such as histones, cytochrome c or calmodulin. This reaction is catalyzed by enzymes known as protein lysyl methyltransferases. The product of this reaction is trimethyllysyl residues which are released from proteins by protein hydrolysis as free trimethyllysine (TML).

Subsequently, TML enters the mitochondria and interacts with the trimethyllysine hydroxylase (TMLD, encoded by the trimethyllysine hydroxylase epsilon gene: TMLHE) which converts TML into 3-hydroxy-N-trimethyllysine[24]. 3-hydroxy-N-trimethyllysine is then cleaved into gamma-trimethylaminobutyraldehyde, a reaction catalyzed by hydroxyl N-trimethyllysine aldolase (HTMLA)[25].

Gamma-trimethylaminobutyraldehyde is then dehydrogenated and forms gamma-butyrobetaine a reaction catalyzed by the 4-trimethylammoniobutyraldehyde dehydrogenase. Finally, L-carnitine is formed by the hydroxylation of gamma-butyrobetaine a reaction catalyzed by the gamma-butyrobetaine hydroxylase (BBOX1).

L-carnitine biosynthesis involves different organelles (the nucleus, the mitochondria, the peroxisome and the cytosol) in various tissues and organs: kidney, liver, brain, *etc*.[26]. Between 1 and 2 μmol of carnitine are synthesized/kg b.w. per day in a human body.

Dietary origin of carnitine

L-carnitine is mainly present in meat and meat products, dairy and fishes provide also a significant amount of carnitine. Most fruits and vegetables are not riche in L-carnitine. An omnivorous diet brings about 50 to 100 mg of carnitine per day, 80% coming from meat while a vegetarian diet brings around 10 mg of carnitine/day.

For regular foods, L-carnitine bioavailability varies from 54% to 87%[27] and for dietary supplements, the bioavailability is only around 15%.

In Human, L-carnitine concentration in muscles is around 3 and 6 μmol of per gram making muscle the major reservoir for L-carnitine in the body, however, it has been shown that L-carnitine present in the muscle does exchange easily with the plasma and muscle is unable to synthesize carnitine and relies on L-carnitine synthesized elsewhere in the body or from the dietary carnitine. In contrast, L-carnitine level in the

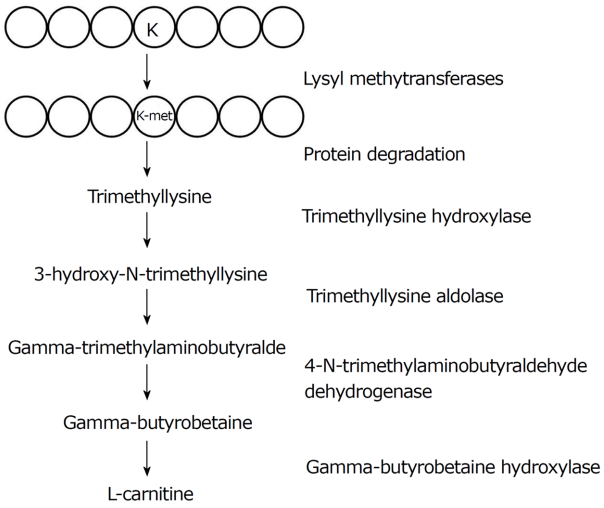

Figure 2 Summarized biosynthetic pathway for L-carnitine. The names of the enzymes are on the right part of the figure.

liver is much less than in the muscle (around 0.5 to 1 μmol of L-carnitine per g of tissue) but the hepatic carnitine can be quickly released in the plasma. High levels of L-carnitine are also found in the testes and the brain.

There is no evidence for a catabolism of L-carnitine in mammalian cells and L-carnitine is eliminated as it from the body in the urine.

Deficiency in human

L-carnitine can be synthesized in several organs (liver, kidney, testis and brain), and can be provided by the foodstuffs. In the human body, carnitine is mainly found in muscles and as muscles are unable to synthesize carnitine they rely on an active transport across the sarcolemma to provide L-carnitine to muscle cells. L-carnitine transport across membrane is done by transporters. The major transporters for L-carnitine belongs to the organic cation transporter (OCTN) family. L-carnitine transport is done through the activity of three transporters: OCTN1 (SLC22A4) and OCTN2 (SLC22A5) in humans and animals and Octn3 (Slc22a21) in mice. A defect in OCTN2 is known to induce primary systemic carnitine deficiency (SCD) a defect that leads to alteration in beta-oxidation of long-chain fatty acids, causing various symptoms, such as myopathy, cardiomyopathy, fatty liver and male infertility[28].

Secondary carnitine deficiency is due to defects in other metabolic pathways or to drugs that impair intestinal or renal absorption of L-carnitine. The consequences of this deficiency are basically the same than those observed in primary deficiency.

Biochemical functions

The major actions of carnitine are due to the fact that carnitine acts as a cofactor for a large family of enzymes: the carnitine acyltransferases. These enzymes are responsible for the esterification of carnitine with acyl groups, allowing the formation of acyl-carnitines. The carnitine acyltransferases are widely distributed in the cell. Carnitine acetyltransferase, is an enzyme that catalyzes the esterification of carnitine into short chain acyl-coenzyme A (acyl-CoA); this activity was described in different organelles: the cytosol, the mitochondrion, the peroxisome and the endoplasmic reticulum in various tissues: the heart, the brain, the kidney, the sperm cells and the liver[29]. Carnitine octanoyltransferase (CrOT) is an enzyme required for the peroxisomal metablism of very long-chain fatty acids and branched-chain fatty acids[30]. Carnitine palmitoyltransferase 1 (CPT 1) is an enzyme located on the outer mitochondrial membrane: it catalyzes the esterification of long chain acyl-coenzyme A to acyl-carnitine. Carnitine palmitoyltransferase 2 (CPT 2) is present in the inner mitochondrial membrane; it catalyzes the conversion of acyl-carnitine back to acyl-coenzyme A and together with CPT 1 and a transporter the carnitine acyl-carnitine translocase allows for the transport of acyl-Co across mitochondrial membranes. Once in the mitochondrial matrix, acyl-CoA can be used in several metabolic pathways: primarily the beta-oxidation.

Mitochondrial metabolism of long chain fatty acids: The mitochondrial metabolism of long chain fatty acids requires several steps: one of them is the entry of fatty acids inside the mitochondria. This transport is done through a system known as the

carnitine system. This 3-protein complex is composed of 2 enzymes: the carnitine palmitoyl transferases 1 and 2 and a transporter: the carnitine acyl-carnitine translocase (CACT). Together these 3 proteins allow the transfer of activated fatty acids from the cytosol to the mitochondrial membrane. In this process, L-carnitine is a key compound that is involved in all the steps of this pathway. Once in the mitochondria matrix, activated fatty acids can enter the beta oxidation pathway and generate energy[31].

Mitochondrial acyl-CoA/free CoA ratio control: Coenzyme A is one of the key compounds in cell physiology and many pathways use this cofactor. Coenzyme A may be present in the cell either as a free compound or bound to various molecules (*e.g*. acyls). Inside the cell, a stable equilibrium between free and acylated CoA exists but this equilibrium may be destabilized. To restore this equilibrium, several process can be activated, one involves L-carnitine. This process involves (1) an increase in acyl-carnitine synthesis which leads to (2) an increase in the mitochondrial beta oxidation. Together these two events induce an increase in the level of free coenzyme and restore the free/acylated coenzyme A ratio[32].

Peroxisomal beta oxidation: In the peroxisome; the β-oxidation of very long chain fatty acids leads to the formation of medium chain-acyl CoAs and acetyl CoA, and is sometimes considered as incomplete contrarily to the mitochondrial beta oxidation which leads to the formation of acetyl-coA only[31]. In the peroxisome, two enzymes dependent on L-carnitine are involve in the beta oxidation of fatty acids. These enzymes are the carnitine acetyltransferase and the carnitine octanoyltransferase. These enzymes seem to be necessary for the exit of medium-chain and short-chain acyl-CoAs from the peroxisomal matrix to the cytosol[30] but the precise role of these enzymes is not clearly defined yet.

Acetylation of histones: Acetylation and deacetylation of histones are crucial mechanisms for the regulation of the transcription. Acetylation requires available acetyl-CoA and it has been suggested that acetyl-carnitine formed in mitochondria[32] can enter the nucleus and be converted into acetyl-CoA and then be used for histone acetylation[33].

Free radical production: Free radicals may interact with various molecular species: lipids, nucleic acids or proteins leading to altered cell function. Several studies have proposed that a dietary supplementation in L-carnitine may exert a protective effect against the deleterious effects of free radicals[34], however, the mechanisms involved in this potential protective effect remain unclear[35].

LINKS BETWEEN AUTISM AND CARNITINE

Although the spectrum of autism is thought to be highly heritable, and may result from multigene susceptibility interactions, no single gene has been identified that can adequately explain the disorder's complex heterogeneity and alarmingly increasing prevalence.

Researches carried out on this topic have pinpointed many genetic variations, including genes involved in carnitine biosynthesis and glutamatergic transmission, which may augment susceptibility to neurodevelopmental disorders like ASD.

The links between L-carnitine and autism rely on 3 major observations (1) the alteration of mitochondrial function occurring in patients with ASD. This aspect has been several times reviewed in the literature and will not be detailed in the present paper (for more information: see ref. [36]); (2) the relationships between L-carnitine levels and the severity of autism and (3) the genetic aspects of autism associated with L-carnitine metabolism. These two aspects are detailed in the present review.

L-carnitine level in ASD patients

A few publications have looked at carnitine levels in patients with ASD and one single publication has studied the effect of a L-carnitine supplementation on ASD patients.

The study of Filipek *et al*[37] measured the level of total and free carnitine in control and ASD patients. They observed that total and free carnitine values were significantly reduced in the autistic individuals with a $P < 0.001$ and that more than 80% of ASD patients have total and free carnitine levels below the reference value. This information was also reported by another group in 2005[38]. Mostafa *et al*[38] also reported a decrease in L-carnitine levels in ASD patients. They described a decrease of almost 50% in L-carnitine levels in ASD individuals. They also reported decreases in lactate levels and poly unsaturated fatty acid levels. This study was done on a

significant number of individuals (30 control *vs* 30 ASD).

Besides this purely quantitative aspect, Frye *et al*[39] analyzed the acyl-carnitine profile of ASD patients, in a large cohort (n = 213), these authors reported that 17% of children with ASD exhibited an elevation in short-chain and long-chain, but not medium-chain, acyl-carnitines. These authors indicated that this pattern of acyl-carnitine abnormalities is similar to what is observed in the brain of propionic acid rodent, model of ASD. This defect in L-carnitine levels is associated with mitochondrial dysfunction.

The acyl-carnitine composition was studied in a population of Chinese children with ASD. The goal of this publication was to identify a possible relation between the acyl-carnitine profile and the intelligence level. This study was carried out on 90 children: 60 with ASD and 30 control. The intelligence level was assessed using the Chinese Wechsler Young Children Scale of Intelligence (C-WYCSI). Blood analysis for L-carnitine derivatives showed that glutaryl carnitine and carnosyl carnitine were significantly decreased in ASD group and the authors of this publication suggested that those 2 compounds may be potential biomarkers for diagnosis of ASD. For these authors, these alterations indicate a potential mitochondrial dysfunction leading to an abnormal fatty acid metabolism in children with ASD[40].

L-carnitine was used as a potential treatment for patients diagnosed with autism. In the study published by Fahmy, 30 children diagnosed with autism (median age 69 mo, ranging from 29 mo to 103 mo) were randomly allotted into either the placebo (n = 14) group or the group receiving 100 mg/kg bodyweight per day of liquid L-carnitine (n = 16) for 6 mo. Several parameters were analyzed in these children: parameters associated with autism such as the childhood autism rating scale (CARS) form and parameters related to carnitine such as free and total carnitine levels. The results presented by the authors revealed a significant improvement in CARS scores in patients receiving L-carnitine, this improvement was associated with an increase in total and free carnitine levels. The authors concluded about (1) the good tolerance of the treatment; and (2) the improvement of autism severity in patient treated for 6 mo with L-carnitine, they also concluded that subsequent studies will be welcome[41].

In conclusion, the analysis of studies carried out on patients with autism for parameters related to L-carnitine suggest that L-carnitine may be lowered in patients with ASD. Furthermore, a L-carnitine supplementation seems to improve the symptoms of autism in patients. However, the relatively low number of publications describing these parameters implies some moderation.

Carnitine synthesis defect and autism

With the overall goal to identify exonic copy number variants in the genome of ASD patients, Celestino-Soper and coauthors[42] analyzed 3743 samples for detecting disease-causing copy number variants (CNVs) that are not detected by most techniques used in conventional research and clinical diagnosis laboratories. This was done on 297 samples from 99 trios (probands with ASD, mother, father). Fifty-five potentially pathogenic CNVs were identified and validated and in a male proband, an exonic deletion of the TMLHE.

Using genome-wide chromosomal analysis methods, Celestino-Soper and coauthors[43] identified 55 potentially pathogenic copy number variants, among those and in the male samples, a deletion of exon 2 of the TMLHE gene (trimethyllysinehydroxylase epsilon) was found, this gene encodes the first enzyme in the biosynthesis of carnitine which is located in mitochondria. The lack of this enzyme leads to a decrease in plasmatic levels of 3-hydroxy-6-N-trimethyllysine and γ-butyrobetaine and in an increase in 6-N-trimethyllysine concentration in the plasma.

In the same time, the entire chromosome X exome was analyzed by next-generation sequencing in 12 unrelated families with ASD affected males. Thirty-six possibly deleterious variants were found located in 33 candidate genes. Among those a mutation in TMLHE, was identified in two brothers with autism. The screening of the TMLHE coding sequence in 501 male patients with ASD allowed the identification of 2 additional missense substitutions. These mutations were shown to induce a loss-of-function and led to an increase in trimethyllysine in the plasma of patients[44].

Based on these observations, a 4-year-old male with a mutation in the TMLHE gene and developing an autism spectrum disorder was supplemented with L-carnitine (200 mg/kg per day). In this young patient, the levels of carnitine were very low. The authors reported that two weeks after the initiation of the treatment, his family reported "noticeable increases in language, non-verbal expression, and engagement with others"[45]. The evolution was positive as the patient's regression ended and even started progressing. In the same time, the levels of carnitine in the plasma increased. Under such circumstances (with a TMLHE deficit) a supplementation in L-carnitine seemed efficient.

More recently, another potential mechanism involving the role of carnitine in the

onset of autism has been propose, it implies a defect in the transport of L-carnitine into the cells. The amino acid transporter SLC7A5, also able to transport carnitine, has been recently shown to be associated with the onset of autism[46]. The same transporter seems also to be implicated in the metabolism of drugs such as Risperidone given for limiting the symptoms in ASD patients. Depending on the polymorphism of the gene, the drug can be differently catabolized[47]. One might notice that the transporter SLC7A5 is not purely a transporter of carnitine but a protein able to transport various amino acids.

CONCLUSION

A few aspects in the relation between carnitine and autism should be highlighted: (1) Low plasma carnitine is reported in autism, but not systematically. Furthermore, it seems that if some affected infants have low plasma carnitine during the early childhood, their plasma carnitine return to normal when measured a few years later[48]; (2) Typically, L-carnitine levels are measured in the plasma, but it is very likely that the important levels of L-carnitine for brain development should be measured in the brain. And little to no information are available on these values; and (3) In any cases, not all patients with ASD have altered levels of carnitine.

In conclusion, some patients with ASD might have L-carnitine synthesis disorders (around 10%-20%) and for these patients, a supplementation with L-carnitine is beneficial. It is still remaining 80%-90% of the patients who have no L-carnitine synthesis or transport defects and for whom the origin of the disease should be find elsewhere.

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MINIREVIEWS

Last decade update for three-finger toxins: Newly emerging structures and biological activities

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Abstract

Three-finger toxins (TFTs) comprise one of largest families of snake venom toxins. While they are principal to and the most toxic components of the venoms of the *Elapidae* snake family, their presence has also been detected in the venoms of snakes from other families. The first TFT, α-bungarotoxin, was discovered almost 50 years ago and has since been used widely as a specific marker of the α7 and muscle-type nicotinic acetylcholine receptors. To date, the number of TFT amino acid sequences deposited in the UniProt Knowledgebase free-access database is more than 700, and new members are being added constantly. Although structural variations among the TFTs are not numerous, several new structures have been discovered recently; these include the disulfide-bound dimers of TFTs and toxins with nonstandard pairing of disulfide bonds. New types of biological activities have also been demonstrated for the well-known TFTs, and research on this topic has become a hot topic of TFT studies. The classic TFTs α-bungarotoxin and α-cobratoxin, for example, have now been shown to inhibit ionotropic receptors of γ-aminobutyric acid, and some muscarinic toxins have been shown to interact with adrenoceptors. New, unexpected activities have been demonstrated for some TFTs as well, such as toxin interaction with interleukin or insulin receptors and even TFT-activated motility of sperm. This minireview provides a summarization of the data that has emerged in the last decade on the TFTs and their activities.

Key words: Three-finger toxins; Snake; Venom; Structure; Biological activity

Core tip: The three-finger toxins (TFTs) of snake venoms are principal to and the most toxic components of elapid venoms. Over 700 TFT amino acid sequences are listed in the UniProt Knowledgebase currently, with new members added constantly. The past decade has also seen multitudinous new discoveries, including structural variations in

TFTs (*i.e.* disulfide-bound dimers), new types of biological activities for the well-known TFTs (*e.g.*, α-bungarotoxin's inhibition of ionotropic receptors of γ-aminobutyric acid), and other new, unexpected activities for the TFTs (*i.e.* interaction with interleukin or insulin receptors and activation of sperm motility). This minireview provides an up-to-date overview of these data.

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INTRODUCTION

Three-finger toxins (TFTs) form an abundant family of nonenzymatic proteins found in snake venoms[1]. The TFTs were so-named according to their characteristic spatial structure, in which three loops (fingers) protrude from the central core, stabilized by four conserved disulfide bonds. The TFTs contain from 57 to 82 amino acid residues, with some toxin types having an extra fifth disulfide bond, located in either their central loop II or N-terminal loop I. The position of the bond affects the toxin's biological activity.

The TFTs manifest a wide array of biological activities, ranging from selective interaction with certain receptor types to nonselective cell lysis[2]. Typically, the TFTs represent the main components of elapid venoms[3]. Thus, in the venom of the desert coral snake *Micrurus tschudii*, 95% of toxins are represented by TFTs[4]. Their presence has also been detected in the venoms of snakes from other families. For example, TFTs were found in different snake genera from colubrid family[5,6]. The TFTs also represent one of the largest families of snake toxins, having more than 700 TFT amino acid sequences deposited in the UniProt Knowledgebase free-access database. Intriguingly, nontoxic structural counterparts of the TFTs have been found in animal organisms, namely the lymphocyte antigen 6 (Ly6) proteins. Based on their commonality of three-finger folding, the TFTs and Ly6 proteins are combined into one Ly6/neurotoxin family[7].

The first TFT discovered, α-bungarotoxin (α-Bgt), was published almost 50 years ago[8]. Since then, α-Bgt has become a widely used specific marker of the α7 and muscle-type nicotinic acetylcholine receptors (nAChRs). In addition, a tremendous number of other TFTs have been discovered, and new members of this family possessing original structures and biological activities are emerging constantly. Moreover, new activities have been recognized for the well-known TFTs, and the discovery of new activities for both the new and the well-known TFTs may be regarded as a recent trend in toxinology.

This minireview briefly summarizes the data obtained for TFTs during the last decade (Table 1). The toxins with new structural features appearing in the recent literature are considered herein; those that have garnered the most interest is the covalently-bound TFT dimers. In addition, the recently discovered uncommon biological activities of some TFTs are discussed; these include the so-called mambalgins that exert a potent analgesic effect upon central and peripheral injection and represent the most remarkable discovery of late.

RECENTLTY-DISCOVERED TFTS WITH NEW STRUCTURAL FEATURES

Ten years ago, the first data revealing covalently-bound TFT dimers were published[9]. Disulfide-bound dimers of TFTs, including heterodimers formed by α-cobratoxin (α-CTX; a long-chain α-neurotoxin) with different cytotoxins and the homodimer of α-CTX were isolated from the *Naja kaouthia* cobra venom. Determination of the homodimer crystal structure allowed identification of the intermolecular disulfides formed by Cys3 in the protomer one and Cys20 in the protomer two, and other way round (Figure 1A). All other disulfides in protomers had the same pairing as in natural α-CTX[10].

The dimerization itself strongly affected the biological activity of the original

Table 1 Novel three-finger toxin biological effects and their potential applications

	Toxin	Effect/Target	Potential application
Impact on signal transduction	Mambalgins	Inhibitors of ASICs	Analgesics
	Micrurotoxins	Modulators of GABAA receptor	Biochemical instruments for the GABAA receptor study
	α-Neurotoxins	Inhibitors of GABAA receptor	
	Muscarinic toxin MTα	Antagonist of α2B-adrenoceptor	Treatment of blood pressure disorders
	Toxin CM-3	Interaction with α1A-adrenoceptor	
	Toxin AdTx1 (ρ-Da1a)	Specific and selective inhibitor for the α1A-adrenoceptor	
	Toxin ρ-Da1b	Antagonist of α2A-adrenoceptors	
	Toxin Tx7335	Potassium channel activator	Biochemical instrument for the study of potassium channels
	Calliotoxin (δ-elapitoxin-Cb1a)	Activator of voltage-gated sodium channel	Biochemical instrument for the study of sodium channels
Impact on blood coagulation	Toxin KT-6.9	Inhibitor of platelet aggregation	Treatment of blood coagulation disorders
	Ringhalexin	Inhibitor of FX activation	
	Exactin	Inhibitor of FX activation	
Insulinotropic activity	Cardiotoxin-I	Induction of insulin secretion from β-cells	Treatment of type 2 diabetes
Impact on sperm motility	Actiflagelin	Activator of sperm motility in vitro	Infertility treatment

ASIC: Acid sensing ion channel; GABAA: Type A receptor of gamma-amino butyric acid; FX: Factor X.

toxins, with the cytotoxic activity of cytotoxins within dimers being completely abolished. However, the dimers were found to retain most of the α-CTX capacity to interact with *Torpedo* and α7 nAChRs as well as with the *Lymnea stagnalis* acetylcholine-binding protein. Moreover, in contrast to the α-CTX monomer, the α-CTX dimer acquired the capacity to interact with α3β2 nAChR, similar to that seen with κ-bungarotoxin (κ-Bgt), a dimer with no disulfides between its monomers (Figure 1D). Collectively, these data show that dimerization of three-fingered neurotoxins is essential for binding to heteromeric α3β2 nAChRs.

In 2009, from venom of the brown cat snake *Boiga irregularis* a new heterodimeric TFT, irditoxin, was isolated[11]. Irditoxin spatial structure determined by X-ray analysis displayed two subunits possessing a three-finger fold, characteristic for nonconventional toxins (Figure 1B). The subunits in the irditoxin dimer are connected by an interchain disulfide bond, formed by extra cysteine residues present in each subunit. In contrast to the dimeric toxins discussed above, irditoxin does not inhibit mouse neuronal α3β2 and α7 nAChRs. However, irditoxin is a bird- and reptile-specific postsynaptic neurotoxin which inhibits the chick muscle nAChR three orders of magnitude more efficiently than the mouse receptor. *In vivo*, it was lethal to birds and lizards and was nontoxic toward mice[11].

Covalently-bound dimers are undoubtedly the most interesting TFT posttranslational modification recently found. A new TFT forming a noncovalent dimer was discovered recently as well; this neurotoxin, haditoxin, was isolated from the venom of king cobra *Ophiophagus hannah*[12]. The high-resolution X-ray analysis revealed that haditoxin is a homodimer (Figure 1C), with a structure very similar to that of κ-Bgt (Figure 1D). However, in contrast to κ-Bgt, the amino acid sequences of the monomeric subunits of haditoxin correspond to those of α-neurotoxins of the short-chain type. It should be noted that κ-Bgt targets the neuronal α3β2 and α4β2 nAChRs and α-neurotoxins of the short-chain type block the muscle-type nAChRs only, while haditoxin demonstrated new pharmacological features, being antagonist toward muscle (αβγδ) and neuronal (α7, α3β2, and α4β2) nAChRs, and having the highest affinity (IC_{50} 180 nmol/L) for α7 nAChRs[12].

The above data indicate that TFT dimerization may underlie the capacity for interaction with neuronal nAChRs.

The disulfide bonds play an essential role in maintaining the spatial structure of TFTs, and their pairing pattern is largely conserved. However, an unusual disulfide bond scaffold was found in a TFT isolated from the venom of eastern green mamba *Dendroaspis angusticeps*[13]. This toxin, named Tx7335, has the highest amino acid sequence similarity to the TFTs of nonconventional type, but differs from them in the number and positions of cysteines (Figure 2). Similar to α-neurotoxins of the short-chain type, Tx7335 has only eight cysteines, while nonconventional toxins have ten

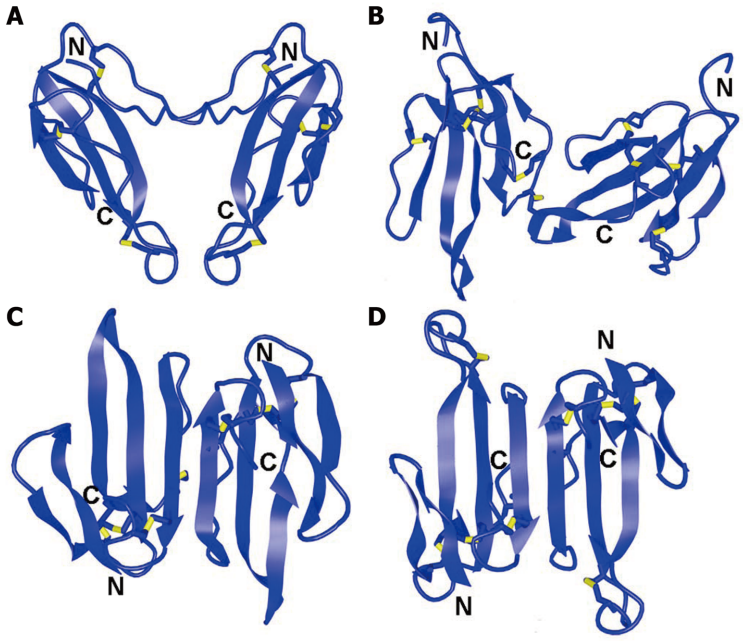

Figure 1 Spatial structures of dimeric three-finger toxins. A: Homodimer of α-cobratoxin, Protein Data Bank Identification code (PDB ID): 4AEA; B: Irditoxin, PDB ID: 2H7Z; C: Haditoxin, PDB ID: 3HH7; D: κ-bungarotoxin, PDB ID: 1KBA. Disulfide bonds are shown in yellow. N and C indicate N- and C-terminus, respectively.

cysteines. Furthermore, Tx7335 contains a tyrosine residue at position 43, which is occupied by a cysteine in all TFTs, while a cysteine is found at position 25. In most other TFTs this position is occupied by a tyrosine (Figure 2). According to structure modeling, Cys55 may make a disulfide bond with Cys25; due to the spatial proximity of these residues no major distortion in the three-finger structure occurs. This novel arrangement of disulfides may explain the unique functional effects observed for Tx7335. Indeed, it has been shown to activate the bacterial pH-gated potassium channel KcsA by a dose-dependent increase in both mean open time and open probability. Moreover, Tx7335 binds at the KcsA extracellular domain at a site probably different from that of channel inhibitors[11].

Several new TFTs retaining the classical arrangement of disulfide bonds but possessing novel structural features have been identified recently. So, from the venom of black mamba *Dendroaspis polylepis polylepis*, a non-typical long-chain TFT, α-elapitoxin-Dpp2d (α-EPTX-Dpp2d), was isolated[14]. α-EPTX-Dpp2d contains an amidated C-terminal arginine, a posttranslational modification that had not been observed before in snake TFTs. Biological activity studies showed that, at a 1 μmol/L concentration, the α-EPTX-Dpp2d potently inhibited neuronal α7 (IC_{50} 58 nmol/L) and muscle-type nAChRs (IC_{50} 114 nmol/L) however showed no effect on α3β2 and α3β4 receptors. Therefore, this amidation was deemed to have no significant effect on the toxin's selectivity, as the activity profile is fairly similar to that of the classic long-chain TFTs with a free carboxyl termini[14].

It was shown that the earlier characterized TFT Oh9-1[15] from *Ophiophagus hannah* venom may represent a new group of competitive nAChR antagonists, known as the Ω-neurotoxins[16]. Electrophysiology experiments on *Xenopus* oocytes showed that Oh9-1 inhibited rat muscle-type α1β1εδ (adult, IC_{50} 3.1 μmol/L) andα1β1γδ (fetal, IC_{50} 5.6 μmol/L) and rat neuronal α3β2 nAChRs (IC_{50} 50.2 μmol/L), but manifested low or no affinity for other human and rat neuronal subtypes. Interestingly, Oh9-1 potentiated the human glycine receptor (homopentamer composed of α1 subunits), with activity increase by about 2-fold. Alanine-scan mutagenesis showed a novel mode of interaction with the ACh binding pocket of nAChRs *via* a set of functional amino acid residues that are different from those in the classical α-neurotoxins. Herewith, the central loop of Oh9-1 interacts with α1β1εδ nAChR by both sides of the β-strand, while only one side of the β-strand interacts with the α3β2 receptor[16].

The taxon-specific dimeric TFT irditoxin, isolated from a rear-fanged snake, was discussed above. Another taxon-specific TFT, fulgimotoxin, was isolated from the venom of the rear-fanged green vine snake *Oxybelis fulgidus*[17]. This toxin is a monomer and contains five disulfides, typical for the nonconventional TFTs. It is highly neurotoxic to lizards; however, mice are unaffected. Similar to other colubrid TFTs, fulgimotoxin has an extended N-terminal amino acid sequence and a

Figure 2 Alignment of amino acid sequence of toxin Tx7335 with those of nonconventional toxins. Cysteine residues are marked in yellow. Black lines indicate the locations of the typical disulfide bond in nonconventional toxins; red line indicates the unusual disulfide bond 25-55 in Tx7335. 3NOJ_DENAN: Toxin Tx7335 from *Dendroaspis angusticeps* (Eastern green mamba); 3NOJ_BUNCA: Bucandin from *Bungarus candidus* (Malayan krait); 3NOJ6_DENJA: Toxin S6C6 from *Dendroaspis jamesoni kaimosae* (Eastern Jameson's mamba); 3NOJ_WALAE: Actiflagelin from *Walterinnesia aegyptia* (desert black snake).

pyroglutamic acid at the N-terminus.

The longest TFT, the nonconventional toxin BMLCL, consisting of 82 amino acid residues and five disulfide bridges, was identified in *Bungarus multicinctus* venom[18]. Earlier studies of biological activity revealed no interaction with the muscarinic acetylcholine receptors (mAChRs) M1 and M2 nor with the muscle-type nAChR[19]. However, recent studies showed that BMLCL interacted efficiently with both α7 (IC_{50} 43 nmol/L) and muscle-type nAChR (IC_{50} 31 nmol/L)[19]. Thus, the longest TFT functions as an antagonist of nAChRs.

It should be noted that so far, no TFTs have been found in the venoms of snakes from the *Viperidae* family; however, transcripts encoding these toxins were identified in venom gland transcriptomes of several *Viperidae* species. To address the question of biological activity of *Viperidae* TFTs, two toxins were obtained by heterologous expression in *Escherichia coli*. Based on the nucleotide sequences of cDNA encoding TFTs in the venom glands of vipers *Azemiops feae* and *Vipera nikolskii*, the corresponding genes optimized for bacterial expression were synthesized[20]. Expressed *A. feae* TFT (TFT-AF) and *V. nikolskii* TFT (VN-TFT), both of the nonconventional type, were refolded under the conditions elaborated on earlier for cobra TFTs. The biological activity of the toxins obtained was studied by electrophysiological techniques, calcium imaging, and radioligand analysis. Both toxins inhibited neuronal α3-containing and muscle-type nAChRs in the micromolar concentration range, but they were each very weak antagonists of neuronal α7 nAChRs. Thus, viper TFTs can function as antagonists of nAChRs of neuronal and muscle-type[20].

RECENTLY DISCOVERED TFTS WITH NEW BIOLOGICAL ACTIVITIES

Novel TFTs affecting signal transduction

The most fascinating biological activity of TFTs discovered during the last decade is their capacity to interact with acid-sensing ion channels (ASICs). ASICs are proton activated and Na^+-selective ion channels, widely distributed throughout the peripheral and central nervous systems (CNS) in vertebrates. ASICs take part in an array of physiological processes, from synaptic plasticity and neurodegeneration to pain sensation. Therefore, the finding of new regulatory modes for these proteins opens up a new avenue of research for pain management, as well as for addiction or fear.

The new mambalgins class of TFTs has been characterized as potent, rapid and reversible inhibitors of ASICs, based on studies with the protein from African black mamba (*Dendroaspis polylepis*) venom[21]. The mambalgins are composed of 57 amino acids and eight cysteine residues, and have about 50% amino acid sequence identity to other snake TFTs. While mambalgins were found to be nontoxic in mice, they were found to exert a potent analgesic effect, as strong as that of morphine but causing much less tolerance than morphine and no respiratory distress. Pharmacological studies showed that mambalgins produce their analgesic effect through the blockade of heteromeric channels containing ASIC1a and ASIC2a subunits in CNS and of channels including ASIC1b subunit in nociceptors. Mambalgins were also shown to inhibit heteromeric channels including ASIC1a and ASIC1b subunits, homomeric rodent and human ASIC1a channels and homomeric rodent ASIC1b channels, the IC_{50}s being in the range from 11 nmol/L to 252 nmol/L[22,23].

The structure of an ASIC1a–mambalgin-1 complex was determined by cryoelectron microscopy at a resolution of 5.4 Å[24]. The data obtained showed that mambalgin-1

binds precisely to the thumb domain of ASIC1a but not to the acid-sensing pocket, as suggested earlier[25]. However, mambalgin-1 binding induced conformational changes in the thumb domain of the channel, which may disturb the sensing of an acidity in ASIC1a[24]. The structural data obtained might provide a structural basis for further development of ASIC modulators.

No less significant than the discovery of mambalgins was the finding of TFTs that interact with ionotropic GABA receptors ($GABA_A$). Almost simultaneously, three research groups found that snake TFTs were able to bind $GABA_A$ receptors[26-28]. Thus, two TFTs, called micrurotoxin 1 (MmTX1) and 2 (MmTX2), were isolated from Costa Rican coral snake (*Micrurus mipartitus*) venom and sequenced[26]. It was shown that at subnanomolar concentrations MmTX1 and MmTX2 increased receptor affinity for the agonist by binding to allosteric site, and thus potentiated opening and macroscopic desensitization of the receptor. The authors suggested that at the molecular level, the α+/β− subunit interface might be involved in toxin action. When injected into mouse brain, both toxins evoked seizures against the background of reduced basal activity[26]. The discovery of toxins enhancing $GABA_A$ receptor sensitivity to agonist established a new class of ligands for this receptor family.

In 2006, it was shown that α-Bgt, a classical blocker of α7 and muscle-type nAChRs, binds to and blocks $GABA_A$ receptors containing the interface of β3/β3 subunit[29]. No effects were observed for α-Bgt on heterooligomeric $GABA_A$ receptors which contain α-, β- and γ-subunits or α-, β- and δ-subunits. However, recently, two research groups independently showed that α-Bgt and some other TFTs could bind to recombinant and native $GABA_A$ receptors[27,28]. Both electrophysiology experiments and fluorescent measurements with α-Bgt coupled to Alexa-Fluor 555 revealed the highest toxin affinity to α2β2γ2 receptor subtype[27]. GABA reduced fluorescent labeling by α-Bgt, suggesting that the α-Bgt binding site overlaps the GABA binding site at the interface of β/α subunits[27].

Binding at the β/α subunit interface was demonstrated for the long-chain α-neurotoxin α-CTX[28], and this toxin interacted more efficiently with the $GABA_A$ receptor than α-Bgt. Electrophysiology experiments showed mixed competitive and noncompetitive α-CTX action, with highest affinity of this toxin being to the α1β3γ2 receptor (IC_{50} 236 nmol/L). Other receptor subtypes were inhibited less potently, as follows: α1β2γ2 ≈ α2β2γ2 > α5β2γ2 > α2β3γ2 and α1β3δ. Among the several TFTs studied, the long α-neurotoxins Ls III (*Laticauda semifasciata*) and neurotoxin I (*Naja oxiana*) as well as the nonconventional toxin WTX (*Naja kaouthia*) interacted with the $GABA_A$ receptor. These data demonstrate that $GABA_A$ receptors are a target for diverse TFTs, including the very well-studied α-Bgt and α-CTX.

Among the vast variety of TFTs there is a class of toxins that interact with mAChRs, which are G-protein coupled receptors (GPCRs)[30]. For many years, the mAChRs were thought to be the only GPCRs affected by TFTs; however, over the last decade, several TFTs capable of interacting with other GPCRs, namely adrenoreceptors of different types, were reported.

It was shown that muscarinic toxin α (MTα) was a more potent antagonist for the α_{2B}-adrenoceptor than for mAChR[31]. MTα inhibited the α_{2B}-adrenoceptor, but did not affect the α_{2A}-, α_{2C}-, α_{1A}- or α_{1B}-adrenoceptors. In ligand binding experiments, MTα superseded the radioligand efficiently (IC_{50} 3.2 nmol/L) and decreased the maximum binding without any influence on the radioligand affinity, demonstrating a noncompetitive inhibition mode[31]. The study of another MT, MTβ, showed nonselective low affinity interaction with the five muscarinic receptor subtypes[32]. Study of the toxin CM-3 (having undefined biological function to date) and MTβ demonstrated high efficacy for α-adrenoceptors and particularly a subnanomolar affinity for the receptor of α_{1A}-subtype[32]. Both toxins were isolated more than 20 years ago from the venom of the African mamba *Dendroaspis polylepis*[33,34]. No or very weak affinity of these toxins were found for muscarinic receptors in the work of Blanchet *et al*[32].

Targeted searches for toxins interacting with α-adrenoceptors have yielded novel information in the last decade. The interactions of fractions obtained from green mamba (*Dendroaspis angusticeps)* venom with α_1-adrenoceptors were tested in binding experiments using ^{3}H-prazosin as a radioligand[35]. A new TFT inhibitor, AdTx1 (renamed later as ρ-Da1a[36]), comprising 65 amino acid residues with four disulfide bridges, was found. ρ-Da1a showed subnanomolar affinity with K_i of 0.35 nmol/L and demonstrated high specificity for the human adrenoceptor of α_{1A}-subtype. Interestingly, the biological activity profile of ρ-Da1a appeared very similar to those of MTβ and CM-3; however, these latter two toxins interacted more potently than ρ-Da1a with α_{1B}- and α_{1D}-adrenoceptor subtypes[32]. ρ-Da1a was, thus, characterized as a specific and selective peptide inhibitor for the α_{1A}-adrenoceptor, acting as a potent relaxant of smooth muscle[35].

Using a similar targeted screening approach, but with application of ^{3}H-

rauwolscine as a radioligand, the effects of venom fractions obtained from green mamba on α_2-adrenoceptors from rat brain synaptosomes were studied[37]. A novel TFT, ρ-Da1b, comprising 66 amino acid residues with four disulfide bridges was isolated. It inhibited binding of ^{3}H-rauwolscine to the three α_2-adrenoceptor subtypes by 80% with affinity in the range of 14-73 nmol/L and with Hill coefficient of about unity. Furthermore, calcium imaging experiments on human α_{2A}-adrenoceptors expressed in mammalian cells showed that ρ-Da1b was an antagonist of this adrenoceptor type[37].

The structural scaffold of aminergic TFTs that are known to interact with various α-adrenergic, muscarinic and dopaminergic receptors was used to generate experimental toxins with new functions. Specifically, the ancestral protein resurrection methodology was applied to identify the functional substitutions that might happen during evolution, and then utilize them for molecular design[38]. Six variants of ancestral toxin (AncTx, 1-6) were generated, and their biological activity was studied. AncTx1 was found to be the toxin possessing to date the highest selectivity to α_{1A}-adrenoceptor. AncTx5 was the strongest inhibitor for α_2-adrenoceptor of the three subtypes. The toxin ρ-Da1a affinities for the α_1- and α_{2C}-adrenoceptor subtypes were modulated most strongly by amino acids at positions 28, 38 and 43 in the evolutionary pathway[38]. Thus, this molecular engineering study represents the first successful attempt to engineer more potent aminergic TFTs.

Among the snake venoms, the mamba ones are unique in their variety of toxins affecting signal transductions[39]. A multitude of toxins capable of disturbing the different stages of cholinergic and adrenergic (see above paragraphs) transmission have been isolated from these venoms. Several toxins affecting voltage-gated ion channels have been isolated as well. The very recently discovered TFT Tx7335, in eastern green mamba *Dendroaspis angusticeps* venom, interacts with the KcsA potassium channel[13]. The unusual structure of this toxin was discussed above. Interestingly, Tx7335 is a channel activator but not an inhibitor, as evidenced by its ability to increase in a dose-dependent mode both mean open times and open probabilities of KcsA incorporated in artificial bilayers; yet, the Tx7335 binding site on KcsA is distinct from that of the canonical pore-blocker toxins. The authors of this study suggested that the toxin allosterically reduced inactivation of KcsA that results in increase of potassium flow through the channel[13].

Blue coral snake *Calliophis bivirgatus* belong to the Elapidae family of snakes the neurotoxic venoms of which typically produce the flaccid paralysis. However it was shown that the *C. bivirgatus* venom uniquely produced spastic paralysis[40]. The toxin producing this paralysis was isolated and called calliotoxin (protein name: δ-elapitoxin-Cb1a). Although calliotoxin is a TFT, it has low amino acid sequence similarity to the other known toxins. It comprises 57 amino acid residues with four disulfide bridges in the classical scaffold. Biological activity studies using HEK293 cells heterologously expressing $Na_V1.4$ showed that the voltage-dependence of channel activation was shifted to more hyperpolarized potentials by calliotoxin. It inhibited inactivation and produced significant ramp currents. These data conformed with profound effects of calliotoxin on contractile force in preparation of isolated skeletal muscle. Thus, calliotoxin represents a functionally novel class of TFTs and is the first activator of voltage-gated sodium channel purified from snake venoms[40].

Novel TFTs affecting blood coagulation

TFTs affecting blood coagulation are not so numerous as those affecting signal transduction. Nevertheless, a new member of the TFT family that is capable of influencing different stages of blood coagulation appeared recently. TFTs inhibiting both primary and secondary hemostasis have been reported.

Primary hemostasis involves platelets, which immediately form a plug at the site of injury. A novel TFT which inhibits the human platelet aggregation process in a dose-dependent manner was purified from cobra *Naja kaouthia* venom and named KT-6.9[41]. KT-6.9 was shown to inhibit platelet aggregation induced by adenosine diphosphate (ADP), thrombin and arachidonic acid but not by collagen and ristocetin. It was 25-times more active than the antiplatelet drug clopidogrel. Based on the data showing significant inhibition (70%) of the platelet aggregation induced by ADP, the authors suggested toxin binding to ADP receptors located on the platelet surface[41].

As for secondary hemostasis, two TFTs capable of inhibiting the extrinsic tenase complex (ETC) were purified from the venom of African ringhals cobra *Hemachatus haemachatus*[42,43]. ETC activates conversion of factor X (FX) to factor Xa (FXa) and represents an important target for the development of novel anticoagulants. A novel TFT anticoagulant, ringhalexin (the ringhals extrinsic tenase complex inhibitor) was shown to inhibit FX activation with an IC_{50} of 123.8 nmol/L[42]. As an inhibitor of mixed type, on chick biventer cervicis muscle preparations ringhalexin manifested an irreversible weak neurotoxicity. The amino acid sequence of ringhalexin is 94%

identical to that of NTL2, an uncharacterized neurotoxin-like protein from *Naja atra*. X-ray crystallography of ringhalexin revealed a typical three-finger structure stabilized by four conserved disulfide bridges[42].

Another novel anticoagulant TFT from *Hemachatus haemachatus* venom, called exactin, can specifically and potently inhibit the activation of FX by ETC (IC_{50} 116.49 nmol/L), similar to ringhalexin[43]. It is also a mixed-type inhibitor of ETC and weakly inhibits FX activation by intrinsic tenase complex (IC_{50} 4.05 μmol/L) and prothrombin activation by prothrombinase complex (IC_{50} 17.66 μmol/L). In contrast to other TFT anticoagulants that are structurally similar to snake cytotoxins, exactin manifests structural similarity to postsynaptic neurotoxins. It also has 82% identity to the weak toxin CM1b from *H. haemachatus* venom and 58% identity to a number of *Ophiophagus hannah* neurotoxins, including the Ω-neurotoxin Oh9-1 discussed above.

Novel TFTs with unexpected biological activities

The last decade has also seen the discovery of several new TFTs possessing quite unusual biological activities.

TFTs, being structurally well defined, thermally stable and resistant to proteolysis, are very good subjects for directed evolution. When a randomization scheme was applied to α-neurotoxin amino acid residues in the loops involved in binding with nAChRs, followed by the cDNA display screening method, new modulators of the interleukin-6 receptor (IL-6R) were obtained[44]. The proteins obtained possessed nanomolar affinity and high specificity for IL-6R. The IL-6-dependent cell proliferation assay revealed both antagonists and agonists in the protein pool. Application of the size minimization procedure resulted in proteins with the molecular mass of about one-third of the original toxin; no significant loss of activities was observed. Moreover, the loops important for function were identified[44]. In another work by the same group, directed evolution was applied to produce a trypsin inhibitor based on the TFT scaffold[45]. The DNA sequences converged after seven rounds of selection. The recombinant proteins obtained were good inhibitors s of trypsin (K_i of 33-450 nmol/L). Three groups of proteins had K_i values close to those of soybean trypsin inhibitor and bovine pancreatic trypsin inhibitor. Two proteins inhibited chymotrypsin and kallikrein as well. The authors suggested that the technique developed may be widely applied for the targeted generation of different regulatory molecules based on the TFT motif[45].

Studies of a TFT cardiotoxin showed a quite unexpected effect on insulin secretion. The fractions of cobra *Naja kaouthia* venom obtained by combination of ultrafiltration and reversed-phase high-performance liquid chromatography were screened for insulinotropic activity using the rat INS-1E β-cell line[46]. Only one fraction of the total 22 obtained induced secretion of insulin from the INS-1E cells with no influence on cell integrity and viability. Liquid chromatography-tandem mass spectrometry analysis revealed that this fraction represented the cardiotoxin-I (CTX-I) isolated earlier from *Naja kaouthia* venom. Analysis of the isolated CTX-I toxin in INS-1E cells showed that its insulin stimulation ability persisted even in the absence of glucose. In contrast to typical cobra cardiotoxin, CTX-I did not induce direct hemolysis of human erythrocytes and showed no potent vasoconstriction capability. Based upon this toxin, a truncated analogue [Lys(52)CTX-I(41-60)] was obtained by structure-guided modification[47]. This analogue showed insulinotropic activity similar to CTX-I and appeared to exert its action through K_v channels[47]. As such, it may serve as a basis for the design of new therapeutic agents for the treatment of type 2 diabetes (Table 1).

A new paradoxical TFT, nakoroxin, was isolated from the cobra *Naja kaouthia* venom[48]. Nakoroxin belongs to the group of orphan TFTs (group "XX"), the biological activities of which are practically unknown. Nakoroxin was not cytotoxic to rat pheochromocytoma PC12 cells nor to human lung carcinoma HT1080 cells. It did not inhibit the binding of α-Bgt to α7 or muscle-type nAChRs, but potentiated the binding of α-Bgt to the acetylcholine-binding protein from *Lymnaea stagnalis*. The reason for this unusual property of nakoroxin is not clear.

Another quite interesting TFT, actiflagelin, was isolated from cobra *Walterinnesia aegyptia* venom by combination of reverse-phase and ion-exchange chromatography[49]. Actiflagelin activated *in vitro* motility of sperm from OF1 male mice. The amino acid sequence established by Edman sequencing combined with tandem mass spectrometry analyses showed that the protein comprised 63 amino acid residues with five disulfide bonds, the pattern of which corresponded to that of nonconventional toxins. Actiflagelin had a noticeable homology to bucandin, a nonconventional toxin from *Bungarus candidus* venom[49]. The authors suggested that the protein found may have therapeutic potential for cases of infertility when the problem is related to the sperm motility.

CONCLUSION

TFTs were among the first toxins isolated from snake venoms. At present, they form one of the largest toxin families and their number is increasing constantly. Several TFTs are used as sophisticated pharmacological tools to study the function and structure of their molecular targets. The new TFTs that have emerged recently possess both novel structural and functional characteristics, expanding the possibilities for their future applications. The TFTs discovered during the last decade have good prospects to be transformed into novel drugs. For example, mambalgins are perfect candidates for the design of powerful analgesics, and on the basis of CTX-I possessing insulinotropic activity, new therapeutics for the treatment of diabetes may be created. The data presented in this minireview (Table 1) show that TFTs continue to be important and promising for both basic science and medicine.

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