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Ion Channel-Target Toxicology

Guest Editors: YongHua Ji, Jan Tytgat,
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Editorial Board

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Editorial
Ion Channel-Target Toxicology

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The saying "use poison as an antidote to poison" has long been considered the spirit of traditional Chinese medicine and herbalism since Ming Dynasty and has indicated the medical values of toxins arising from venomous animals, plants, microbes, or synthetic chemicals. A well-known physician Sir P. M. Latham (1789–1875) once defined the relationship between poisons and medicines as "oftentimes the same substances given with different intents", what highlighted the double-edged sword nature of toxins when applied with different doses.

In view of the great potential of toxins as medicines and drug precursors, a stormy survey and pursuit has been implemented within pharmaceutical industry and research centers in a worldwide range. A classical example comes from the studies of a Brazilian scientist, Dr. Sergio Ferreira, who discovered, in the 1960s, bradykinin potentiating peptides (BPPs), extracted from the venom of the Brazilian viper *Bothrops jararaca*. These peptides were later shown to be able to decrease arterial pressure. This study was very important for the development of an analogue molecule, by Squibb Laboratory, called Captopril, one of the most successful examples of blockbuster drugs, used as an angiotensin converting enzyme inhibitor (ACEi), currently used in the treatment of hypertension, generating more than 8 billion dollars a year.

Nowadays, a representative instance comes from the successfully commercialized toxin product "Ziconotide," developed by Eli Lilly Incorporated (USA), from the venom of *Conus*. On the other side, JZMed Incorporated, a worldwide renowned market research firm specialized in the Chinese pharmaceutical industry, once forecasted that the Chinese preclinical and toxicology outsourcing industry would likely grow in a compound annual growth rate of 27% in five years after 2010 and its market value would likely reach more than $760 million by 2015.

Despite the rapid growth and worldwide attention to toxin R&D industries, it has to be well recognized that the productivity of discovering a new medicine from toxins is still little efficient in the global biopharmaceutical industry. The hindrances toward toxin-drug transformation may include but not be limited to (1) overall costs of drug development; (2) elimination of nondevelopable drug candidates; (3) extensive toxicology and safety pharmacology researches for any drug candidates. Among them, one of the major challenges of toxicity evaluation and integrated efficacy may be the targets of toxins in the human body.

Up to now, it has been well documented that ion channels are specific targets for numerous toxins. Neurotoxins secreted by various venomous species have played critical roles in understanding the physiological contributions of ion channels to the neuronal network as well as in probing and correlating ion channel structure and function. Ion channel-targeted toxins have been validated as promising drug candidates or leading molecules for the treatment of various neuronal syndromes.

All the above considerations led us to gather the current state of the art on natural toxins or toxic compounds targeted to ion channels with possible applications in health care and biotechnology.

This special issue provides a timely focus on the recent progress in our understanding of the mechanisms of toxins specially involved in neuronal dysfunctions. Geographically dispersed excellent specialists in this area from China, Brazil, USA, and France contributed to this special issue with 6 papers: 5 reviews and 1 full research article, covering from

structure-function relationship to the neurological diseases involved with ion channel-targeted toxins, including the study of novel animal models. Although this special issue is a first attempt in the field of ion channel-targeted toxicology, a subsequent and more expanded issue with complementary topics shall be released in the near future.

The development in the field of toxinology owes much to the use of various nervous preparations obtained from various animal models. Among them, the biophysical principles of the nervous system function in insects have been shown to be the same as in mammals. In both groups of animals, similar neurotransmitters can be found, although their distribution varies. M. Stankiewicz et al. present the role of the nervous system of the cockroach *Periplaneta americana* as a well-addressed preparation in the development of toxinological studies. In addition, they provide a systematic introduction to electrophysiological methods to be applied therewith, which allow us to perform pharmacological tests in various levels of the nervous system organization. They also reconsider the definite statement concerning the mode of action of any toxin when carrying out experiments under different conditions.

Considered fundamental in many physiological processes, K^+-channels are recognized as potential therapeutic targets in the treatment of several central nervous system diseases. C. D. C. Gati et al. provide an overview of CNS K^+-channels involved in memory acquisition and storage and evaluate the use of highly selective K^+-channel blockers derived from arthropod venoms as potential therapeutic agents for CNS diseases involving learning and memory mechanisms. Particularly, M.-F. Martin-Eauclaire and P. E. Bougis summarized recent work on the molecular mechanism of toxin-channel interactions of several high-affinity blockers selective to various K^+ channels (SK_{Ca}, $K_v4.x$, and $K_v1.x$ K^+ channel families), from the venom of the Moroccan scorpion *Androctonus mauretanicus mauretanicus,* which may provide new insights into the targets and the mode of action of KTxs.

Even though the studies of ion channel structure-function relationship have always been the most pursued spots, the posttranslational modification processes, such as glycosylation, phosphorylation, and alternative splicing associated with channel functions, have also been investigated. Z. Liu et al. proposed that sodium channel-specific neurotoxic toxins, a family of long-chain polypeptides originating from venomous animals, potentially share binding sites adjacent to glycosylated regions of VGSCs (voltage gated sodium channels). Thus, an interaction between toxins and glycosylated VGSCs might hopefully join the campaign to approach the role of glycosylation in modulating VGSC-involved neuronal network activity.

Apart from voltage-gated ion channels, ionotropic glutamate receptors classified as ligand-gated ion channels, such as NMDA, AMPA, and kainate receptors, are also thought to mediate much of the excitatory neurotransmission in the brain. D. R. Morris and C. W. Levenson discussed the role of the excitotoxic influx and zinc accumulation, the mechanisms responsible for its cytotoxicity, and a number of disorders of the central nervous system linked to these neuronal ion channels and zinc toxicity. The in-depth researches in toxic chemicals targeted on neuronal receptors may hopefully help to develop strategies to block zinc-mediated damage and prevent undesirable outcomes.

A very interesting study was developed by L. C. Carrijo-Carvalho et al., who discovered a peptide believed to be involved in development, regeneration, and pathological processes. It is abundant in the venom of the caterpillar *Lonomia obliqua*. Notably, this research evaluated the effects of this peptide based on lipocalin motif in human fibroblasts in morphogenesis and tissue homeostasis. This was the first paper of lipocalins modulating fibroblasts and ECM proteins.

We extremely appreciated the great contributions of the researchers who have warmly participated in this special issue as writers or reviewers. It is our best wish that the special issue presented here incites new studies that provide a better understanding of the mechanisms underlying ion channel-targeted toxicology, eventually leading to more effective treatments with toxin-evoked epidemiology or other health care concerns.

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Review Article

Mining the Virgin Land of Neurotoxicology: A Novel Paradigm of Neurotoxic Peptides Action on Glycosylated Voltage-Gated Sodium Channels

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Voltage-gated sodium channels (VGSCs) are important membrane protein carrying on the molecular basis for action potentials (AP) in neuronal firings. Even though the structure-function studies were the most pursued spots, the posttranslation modification processes, such as glycosylation, phosphorylation, and alternative splicing associating with channel functions captured less eyesights. The accumulative research suggested an interaction between the sialic acids chains and ion-permeable pores, giving rise to subtle but significant impacts on channel gating. Sodium channel-specific neurotoxic toxins, a family of long-chain polypeptides originated from venomous animals, are found to potentially share the binding sites adjacent to glycosylated region on VGSCs. Thus, an interaction between toxin and glycosylated VGSC might hopefully join the campaign to approach the role of glycosylation in modulating VGSCs-involved neuronal network activity. This paper will cover the state-of-the-art advances of researches on glycosylation-mediated VGSCs function and the possible underlying mechanisms of interactions between toxin and glycosylated VGSCs, which may therefore, fulfill the knowledge in identifying the pharmacological targets and therapeutic values of VGSCs.

1. Introduction

In neurons and most excitable cells, multiform action potentials driven by depolarizing neuronal firing are considered to be accounted by spatiotemporal activation and integral performances of tissue-specific VGSCs [1]. Generally, VGSCs consist of an α subunit (260 kDa) and several auxiliary β subunits (β1–β4, 33–38 kDa). The α subunit is well organized in four homologous domains (DI–DIV) which contain six transmembrane segments each (S1–S6). The hairpin-like loop between S5 and S6 segments region is functioned as ion-permeable pore [2]. Notably, both α and β subunits are highly glycosylated cross-membrane proteins [3–7] (Figure 1).

The most common form of glycosylation sites on VGSCs protein is mainly composed by N-linked sialic acids, [8]. Estimation indicates that 15%–40% of the total VGSC α subunit molecular weight is carbohydrate [6, 7, 9]. Approximately 40%–45% of the added carbohydrate residues are sialic acid moieties, resulting in the addition of an estimated 100 sialic acid residues per subunit molecule [6, 9]. Up to now, through bioinformatics prediction, there have been found tens of potential extracellular glycosylation sites located mainly within the pore region lining between DI S5-S6 in α subunit of VGSCs, less of which, however, have been funtionally characterized [5, 10, 11]. Far less than α subunit, only three of the four N-linked glycosylation sites present in the N terminus of β subunits are thought to be glycosylated in the mature protein [5, 12].

Glycosylation has long be known to participate in regulation of functional expression of channels, such as folding, trafficking, and membrane-insert localization, but also influence electrophysiological properties [13–16]. Inhibition of N-linked glycosylation altered the voltage dependence of channel gating of $K_v1.1$ and K_vLQT/minK (IsK) and the open probability of the renal outer medullary K^+ channel ROMK1 (inward rectifier K^+ channel), as well as the pH sensitivity of K_vLQT/minK channels [17, 18]. In addition, N-linked glycosylation was found to be capable of increasing the stability of Shaker potassium channels proteins in trafficking from the endoplasmatic reticulum to the Golgi [13, 15]. Comparably, the information of glycosylation on modulating

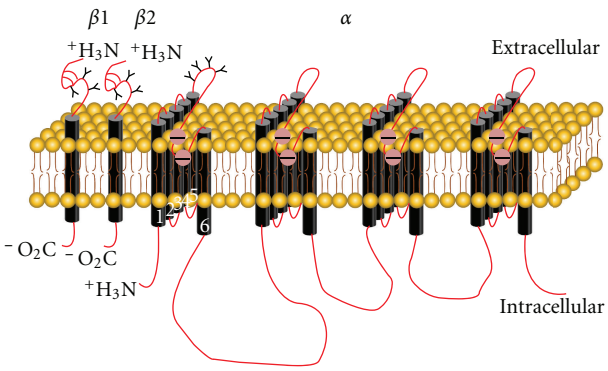

Figure 1: Structure and localization of glycosylation sites of VGSC. The primary structures of the subunits of the voltage-gated ion channels are illustrated as transmembrane-folding diagrams. Cylinders represent probable α-helical segments. Bold lines in red represent the polypeptide chains of each subunit, with length approximately proportional to the number of amino acid residues in the brain sodium channel subtypes. The extracellular domains of the β1 and β2 subunits are shown as immunoglobulin-like folds. Sites of probable N-linked glycosylation; open circle with "—", amino residues that form the ion selectivity filter and tetrodotoxin binding site.

VGSCs function was less approached. The current knowledge about the modulation of glycosylation on VGSCs is mainly focused on reducing the voltage-dependent gating sensitivity, and thereby lowering the AP threshold at neuronal network level [19–21]. But questions like (1) how does glycosylation control the local static milieu on the surface of VGSCs protein which is attributed to the state of cell membrane? (2) what about the subtype-specific modulation of glycosylation on VGSCs? (3) does each glycosylation site modulate equally or synergically on the VGSCs gating? still appear as difficult tasks to work out.

To address the above problems, one may resort to segment-swap chimera construction or glycosylation-deficient cells to reduce the level of glycosylation [10, 22]. However, such methods may still bring about the unexpected artificial factors. More importantly, the complexity of extracellular environment and structure of channel protein itself may also disturb the reliability. Therefore, one prospective and efficient way is to find out the subtype-specific glycosylation modulators.

Natural toxic polypeptides originated from various venomous animals are deemed to be specifically targeting on VGSCs by either lowering the threshold for activation or delaying the inactivation process [23–26]. To date, there have been found six receptor sites of these toxins on VGSCs, some of which are even residing in the overlapped region of glycosylated sites on VGSCs [23, 26–28]. Meanwhile, the pharmacological studies have demonstrated that the binding of these toxins and their targets is highly subtype specific [26, 27, 29]. Thus, they are hopefully utilized as efficient tools to more precisely uncover the role of glycosylation on VGSCs gating and the overall performances on channel pathology in clinical therapy.

2. Mechanism of Glycosylation to the Voltage Dependence of VGSCs Gating

A general understanding about the physiological function of glycosylation on VGSCs is to control the voltage sensitivity in a channel through the number of sialic acids residing on [30]. Once these sialic acids are removed by deglycosylated reagents, such as tunicamycin and neuraminidase, the voltage-dependent activation will shift to a more depolarized direction and thereby raising the threshold for AP generation [7, 22]. Currently, one commonly accepted notion is that it was negative charges brought about by a significant content of sialic acid residues on the glycosylation sites of the extracellular region that caused the hyperpolarized voltage dependence of gating [22]. However, some reports have suggested that various $Na_v\alpha$ subunits are differently glycosylated/sialylated even expressed in the same cell line [19]. This difference in α subunit sialylation directly and differently alters channel gating [10]. Here, two mechanisms describing the differential modulation of glycosylation on VGSCs gating are discussed below.

2.1. "Subtype-Specific" Mechanism. It is well known that VGSCs have nine tissue or developmentally distinct subtypes, named as $Na_v1.1$ to $Na_v1.9$, each of which has been found to be responsible for functionally diverse electric activities within the specific region they are expressed [31]. Recent researches have indicated that different VGSC subtypes have differential responses to the glycosylation.

Deglycosylated $Na_v1.4$ could result in a depolarizing shift in both voltage-dependent activation and inactivation. Comparatively, deglycosylated $Na_v1.5$ could lead to a depolarized voltage-dependent activation but not inactivation [20, 21]. By contrast, deglycosylation could only shift the midpoint of steady-state inactivation of $Na_v1.9$ in adult small DRG neurons to more depolarized potentials [20]. Gating of $Na_v1.2$ and $Na_v1.7$ could not be significantly affected by deglycosylation [32]. Our recent work found that the steady-state activation curve of deglycosylated $Na_v1.3$ was depolarized to a more positive direction, while inactivation curve was negatively shifted [33]. Thus far, glycosylation is capable of modulating VGSCs gating to various extents.

There was a report which attributed such differential modulation of glycosylation to distinct extent of glycosylation of each VGSC subtype. Immunoblot data suggested that $Na_v1.4$ is more glycosylated than $Na_v1.5$ [10]. As a matter of fact, $Na_v1.1$–$Na_v1.4$, are heavily glycosylated (about 15~30%), whilst $Na_v1.5$ and $Na_v1.9$ are barely glycosylated (~5%) [20]. Accordingly, it was indicated that $Na_v1.4$ voltage-dependent gating parameters are significantly and essentially uniformly altered by sialic acid than that of $Na_v1.5$. For the reason that the external surface of one VGSC α-subunit was estimated to have about 110–130 negative charges composed by the sialic acids (amount to about 40% of total carbohydrate in VGSC) [32], it is likely that sialic acid alters the electric field sensed by the gating mechanism of the channel [10]. That is, a higher level of glycosylation

in VGSC may lead to more depolarized shift in voltage-dependent gating when deglycosylated. As a consequence, the deglycosylated VGSCs may require larger depolarizing stimulus to activate.

2.2. "Cell-Specific" Mechanism. One seemly contradictory notion against the "subtype-specific" mechanism was that it was because of certain internal environment of different cells types which differentially produce sialylated proteins leading to a spectrum of Na_v functional sialic acid levels that directly modulate channel gating. Hence, such mechanism was termed as "cell specific" [19, 32].

"Cell-specific" may arise from two compensated factors. One is that the glycosylation extent in certain VGSC protein may vary throughout the developmental stages: for example, Na^+ currents in adult rat cortical and dorsal root ganglion neurons are less sensitive to sialic acid than Na^+ currents from neonatal neurons [20, 34]. Adult ventricular myocyte VGSCs were more heavily sialylated and gated at more hyperpolarized potentials than they were in neonate ventricular myocyte VGSCs. One possible explanation for this increased sialylation would be a chronic increase in sialyltransferase activity in the developing ventricles [19].

Another aspect resulting in differential glycosylation came from the tissue- or cell-type-specific proteins that modify the number of sialic acids on the surface of VGSCs. One recent work have suggested that the modulation of glycosylation may display a rather complex profile with the combinatorial link with VGSC β-subunit: for example, when $\beta 1$ subunit was coexpressed with $Na_v1.2$, $Na_v1.4$, $Na_v1.5$, and $Na_v1.7$, the extent of hyperpolarized shift in voltage-dependent activation has the following order: $Na_v1.7 > Na_v1.5 > Na_v1.2 > Na_v1.4$, where $Na_v1.4$ was less modulated by $\beta 1$ subunit, which indicated an essentially saturating level of functional sialic acids. On the contrary, $Na_v1.7$ alone contain the least functional sialic acids in DIS5-S6, and therefore the levels of functional sialic acids increase, causing the most remarkably hyperpolarization in voltage-dependent activation when coexpressed with $\beta 1$ subunit [32].

3. Novel Paradigm of Interaction between VGSCs and Neurotoxic Peptides

Since the distinct binding affinity with VGSCs, natural toxins such as that from marine animals (saxitoxin, sea anemone) and arthropods (scorpion toxins, spider toxins) have long been applied to investigate the structure-function relationship of VGSCs and regarded as pharmacological templates for developing therapeutic leads [23–26, 35].

However, there have been paradoxes seen in contact cells and in vitro studies on VGSCs. For instance, BmK I, a site-3-specific modulator of VGSCs from scorpion *Buthus martensii* Karsch (BmK) was capable to prevent the inactivation of $Na_v1.2$ and produce persistent current, which may account for BmK I-induced epileptiform responses in rats [36–39]. However, the specific binding of BmK I to the VGSCs in rat brain synaptosomes (mainly $Na_v1.2$) was undetectable [40]. Although the contradictory modulation of BmK I between VGSCs-rich synaptosomes and heterologously expressed $Na_v1.2$ may somewhat attribute to lack of BmK I-sensitive VGSCs [37], the complex intracellular enzymatic context of synaptosomes stimulated us to speculate the involvement of glycosylation on modulating sensitivity of VGSCs to BmK I.

In our recent researches, it was found that BmK I could distinctively modulate glycosylated/deglycosylated $Na_v1.2$ expressed in oocytes. The voltage dependent activation of deglycosylated $Na_v1.2$ was significantly shifted to more negative direction by BmK I, contrary to the little effects on the glycosylated ones. This research indicated that the glycosylated sites on VGSCs may act as umbrellas to shield the interaction sites of VGSCs with BmK I, and if biochemically removed, may facilitate the binding of BmK I to the receptor sites (Figure 2).

Coincidentally, as a receptor site-3-specific modulator, BmK I has been suggested to be capable of binding to the region where glycosylation sites also reside [37, 40]. It has been indicated that the extracellular loops between transmembrane segments S5 and S6 in domain I of the α-subunit are involved in the formation of receptor site 3 [28]. In addition, several antibodies that recognize the extracellular loops between transmembrane S5 and S6 in domains I and IV prevent α-scorpion toxin binding to receptor site 3, suggesting involvement of amino acid residues in these locations [41]. All these clues may potentially support the notion that there exists interaction between glycosylation sites and BmK I, by which the binding affinities of BmK I to VGSCs may be altered if the channel was decosylated.

Similar phenomenon can also be referred to the mode of actions of sulfamethoxazole (SMX) on HERG channel accompanying with a mutant subunit MiRP1 (T8A). Under normal conditions, the carbohydrates were attached to shield the variable receptor and thus impairing the SMX binding. Conversely, in channels formed with MiRP1 T8A mutants that are deficient in glycosylated sites by mutation, SMX accessibility to the receptor is facilitated by the absence of the oligosaccharide groups [42].

Likewise, Catterall et al. in 1987 have concluded the possible mechanism to explain the lack of binding of saxitoxin, one VGSCs-specific blocker, to the glycosylated VGSCs: the negative surface charges on the glycosylated sodium channel are expected to increase the local concentration of Na^+ near the extracellular opening of its transmembrane pore and to increase the local concentration of a cationic ligand like saxitoxin near its receptor site. The lack of effect of inhibition of sialylation with castanospermine on saxitoxin binding indicates that the saxitoxin receptor site is located distance from the negative surface charges contributed by sialic acid residues or is insulated from their effects by the protein structure [7].

4. Perspectives

Several lines of evidence suggest that regulation of the level of sialylation is a powerful mechanism to control the surface charge of channels as well as neuronal channel pathology [27, 43–45]: for example, long QT syndrome

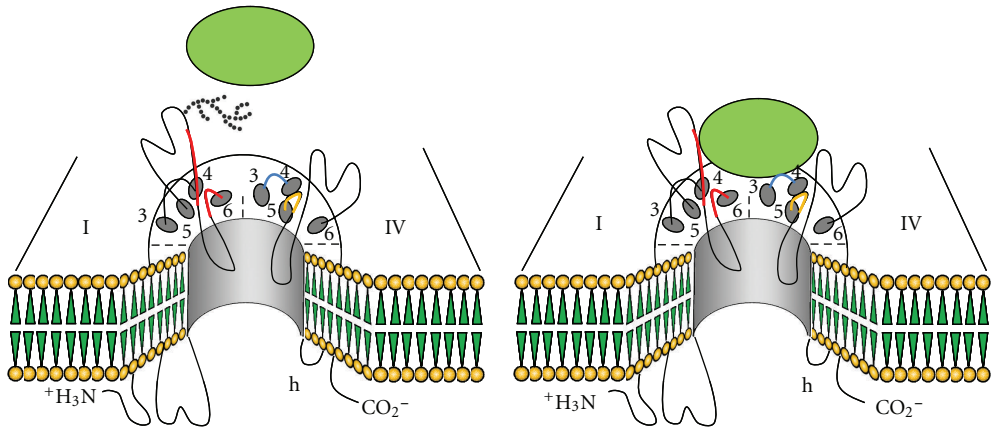

Figure 2: Hypothesis on deglycosylation-created modulation of scorpion toxins to VGSCs. For glycosylated VGSC, the sialic carbohydrates (dotted branch) shield the binding site and thus prevent toxin binding (left). If the sialic carbohydrates are deleted, the toxin accessibility to the binding site is facilitated because of the absence of the oligosaccharide groups and consequently modulate the gating of VGSCs (right).

(LQT, a cardiac disease relate to HERG) is also caused by the mutation of glycosylated sites in HERG [45]. It was reported that glycosylation is essential for the biosynthesis and the maintenance of functional VGSCs in fibroblastoma cells [46]. Additionally, one of main pathogenesis resulting in sinus arrhythmia is insufficient level of glycosylation in cardiac VGSCs [47]. Moreover, some studies showed that the chronic pain is related to the increase of glycosylation in neuron membrane in nerve injury sites [48]. Finally, an increase in sialic acid-negative surface charge resulting in reduced AP threshold and increased excitability could be one of the important factors of the pathogenesis of epilepsy associated with these inherited disorders [22].

The researches on detecting the modulation of glycosylation on VGSCs function are subjected to two major challenges. (1) There still lack of specific tools to precisely and efficiently modify the level of glycosylation on VGSCs, which may result in ambiguous observations in the real studies. Maybe some bioinformatics predictions could provide the auxiliary clues to compensate this problem. However, it remains to be more cautious in treating the computer-assisted data even until some site-directed evidence has been conducted. (2) The static alteration in glycosylation sites can restructure the receptor sites in VGSCs where some potential specific drugs may bind with. Thus, it is a critical task to discriminate the possible disturbance of glycosylation when treating with some seemly inefficient drugs targeting on VGSCs.

As sodium channel-specific modulators and neurotoxins have great potentials to probe the intriguing subtle structural variations in VGSCs found across different tissues and species. However, the pharmacological sensitivity of VGSCs toward toxins, seen in Section 3, may be subject to differential modulation due to the glycosylation of VGSC protein itself, leading to the difficulties in observing the actual interactions. Hence, a broader view of how neurotoxins modulate neuronal activities and thereby valuable information regarding to VGSCs as therapeutic targets could be obtained until more precise details about the role of glycosylation in determining toxin-channel interaction be deduced.

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Research Article

A Lipocalin-Derived Peptide Modulating Fibroblasts and Extracellular Matrix Proteins

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Lipocalin family members have been implicated in development, regeneration, and pathological processes, but their roles are unclear. Interestingly, these proteins are found abundant in the venom of the *Lonomia obliqua* caterpillar. Lipocalins are β-barrel proteins, which have three conserved motifs in their amino acid sequence. One of these motifs was shown to be a sequence signature involved in cell modulation. The aim of this study is to investigate the effects of a synthetic peptide comprising the lipocalin sequence motif in fibroblasts. This peptide suppressed caspase 3 activity and upregulated Bcl-2 and Ki-67, but did not interfere with GPCR calcium mobilization. Fibroblast responses also involved increased expression of proinflammatory mediators. Increase of extracellular matrix proteins, such as collagen, fibronectin, and tenascin, was observed. Increase in collagen content was also observed in vivo. Results indicate that modulation effects displayed by lipocalins through this sequence motif involve cell survival, extracellular matrix remodeling, and cytokine signaling. Such effects can be related to the lipocalin roles in disease, development, and tissue repair.

1. Introduction

Development and regeneration are processes driven by dynamic regulation of extracellular matrix (ECM). ECM is continuously exposed to physical and chemical injuries, and its composing proteins are continuously synthesized and secreted by fibroblasts, which play a central role in regulation of tissue homeostasis. Thus, in young and healthy tissue there is a balance between ECM deposition and degradation, in a well-organized and regulated process [1, 2].

Dysfunctions in deposition and remodeling of ECM proteins hinder normal tissue repair and are observed in several pathologies, such as chronic wound [3], sclerosis and other fibrotic diseases [4, 5], tendinopathy [6], diabetes [7], renal disease [8], pulmonary disorders [9], and even heart disease [10]. Many of these have involvement of cytokines. In addition, other undesired conditions such as chrono- and photoaging are associated with breakdown and impaired synthesis of ECM proteins, especially collagen [2].

Interestingly, lipocalin levels are particularly elevated in some of these and other pathological states [11–15], as well as in site-specific injuries [16–18]. Furthermore, expression of lipocalins has been associated with regeneration and tissue repair [16–20], metamorphosis [16, 21, 22], pregnancy [23], chondrogenesis [24, 25], and other processes related to embryogenesis and postnatal development [14, 24, 26–28]. These findings suggest those proteins play a special role in morphogenesis. Lipocalin roles may be beyond their particular lipophilic ligand-binding properties, given the broad phylogenetic range and tissue distribution of lipocalins reported in these studies.

Lipocalins are among the most abundant proteins found in the venom of the *Lonomia obliqua* caterpillar [29, 30].

They are multifunctional proteins with a β-barrel structure, which share three characteristic conserved domains in their primary structure, namely, motifs 1–3 [31, 32]. The involvement of motif 2 in cell modulation displayed by lipocalins has been previously demonstrated through a peptide mapping approach studying a toxin from *L. obliqua* [33]. In this study, we investigated the effects of a peptide based on this lipocalin motif on human fibroblasts, evaluating the extracellular matrix proteins in vitro and in vivo, mobilization of intracellular calcium, and mediators involved in cell response.

2. Materials and Methods

2.1. Reagents and Antibodies. Ham's F-12 culture medium was purchased from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) and trypsin-EDTA were from Cultilab (Campinas, SP, Brazil). Vectashield mounting medium was from Vector Laboratories (Burlingame, CA, USA). Monoclonal antibodies to cellular fibronectin, human tenascin, and heat shock protein 47 (HSP47) were from Sigma-Aldrich (St. Louis, MO, USA). Other mouse IgG antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA). Alexa Fluor 488 goat anti-mouse IgG antibody was from Molecular Probes (Eugene, OR, USA). FLIPR Calcium 4 Assay Kit was obtained from Molecular Devices (Sunnyvale, CA, USA). All other reagents were supplied by Sigma-Aldrich (St. Louis, MO, USA).

2.2. Peptide Synthesis. Lipocalin motif-2-derived peptide (pm2b) [33], with the amino acid sequence YAIGYSCKDYK-OH, was obtained in automated benchtop simultaneous multiple solid-phase synthesizer, PSSM 8 system (Shimadzu, Kyoto, Japan) using Fmoc solid phase. The peptide was purified by reversed-phase chromatography semipreparative HPLC (Shim-pack Prep-ODS, Shimadzu), and its purity and identity were confirmed by LC-MS mass spectrometry Surveyor MSQ Plus, Thermo Fisher Scientific (San Jose, CA, USA) and by analytical HPLC.

2.3. Cell Culture. Cultures of primary human fibroblasts were obtained from skin biopsies. Cells were grown in Ham's F-12 medium, supplemented with FBS (15%), ampicillin (20 mg/mL), streptomycin (20 mg/mL), and gentamicin (40 mg/mL), at 37°C in a humidified 5% CO_2 incubator. Having reached confluence, cells were washed three times with phosphate-buffered saline (PBS), detached by mild treatment with trypsin-EDTA, and washed with FBS-supplemented medium. Experiments were carried out with cells from the second passage. To evaluate ECM proteins, fibroblasts (1.15×10^5 cells) were cultured on round slides in 24-well plates incubated with pm2b for 96 h in 500 μL of medium. To obtain cell lysates, fibroblasts were cultured in 25 cm^2 culture flasks and incubated with pm2b for 96 h. For flow cytometry analysis, fibroblasts (1.5×10^5 cells/mL) were incubated for 72 h with pm2b in 10% or 1% FBS-supplemented medium. All experiments, unless indicated, were carried out with the peptide at 230 nM. Peptide vehicle (150 mM NaCl) was used as control of treatments.

2.4. Immunocytochemical Staining for ECM Proteins. Cells were gently washed with PBS and fixed for 15 min in 3% paraformaldehyde, 0.2% glutaraldehyde, 0.1 M phosphate buffer, pH 7.4. Then, slides were washed with PBS and subjected to immunostaining with monoclonal antibody anticellular fibronectin, anti-HSP47, or anti-human tenascin and secondary antibody Alexa Fluor 488, according to manufacturer's instructions. Cells were washed and slides mounted with Vectashield. Slides were visualized under fluorescence microscopy (Carl Zeiss, Jena, Germany) by 200x magnification, and ten microscopic fields were analysed. Expressions of procollagen, fibronectin, and tenascin were quantified through morphometric analysis and digital densitometry using an Image System Analyzer (Kontron Electronic 300, Zeiss). Values were normalized to untreated controls.

2.5. Western Blotting. Cells were lysed in RIPA buffer for 20 min at 4°C and centrifuged for 5 min at 21,000 g, to obtain soluble and insoluble extracts. The cell lysate of treated (77 and 230 nM pm2b) and control fibroblasts were subjected to SDS-PAGE, using 30 μg of each protein sample of soluble extracts. Fibronectin and laminin were analyzed using the insoluble extracts. Then, proteins were electrotransferred to nitrocellulose membrane, which was blocked with 1% bovine serum albumin in 20 mM Tris-HCl pH 7.4, 0.15 M NaCl, and 0.05% Tween (TBS-T) and incubated in the same buffer with primary antibodies for fibronectin, laminin, collagen type I, HSP47, and GADH as control. Membranes were washed with TBS-T and incubated with secondary antibody conjugated with alkaline phosphatase. Incubation with each antibody was according to manufacturer's instructions. Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) substrates were used for immunostaining. Proteins were quantified through digital densitometry using the ImageJ software (National Institutes of Health, USA). Values were normalized to untreated controls.

2.6. In Vivo Treatment. BALB/c mice (20 ± 2 g) were bred at Butantan Institute. Animals had free access to food and water and were in a light-dark cycle of 12 h. Mice were anesthetized with ketamine (75 mg/kg) and xylazine (10 mg/kg) i.p. and had the dorsum shaved. The animals were divided into 2 groups, treated with intradermal injections of a single dose of pm2b (0.2 mL, 1.15 μM, $n = 6$) or two repeated doses (7 days of interval, $n = 4$) in a delimited site on the dorsum. Paired controls were injected with the vehicle (saline) in a delimited site on the opposite side in the same animal. Skin fragments of 1 × 1 cm from each site (pm2b-treated and control) were collected in pairs of treated mice in intervals of one, two, and twelve weeks after single treatment, as well as one and twelve weeks after the first dose of repeated treatment. Skin samples were immediately fixed in 10% buffered-formalin for histological procedures.

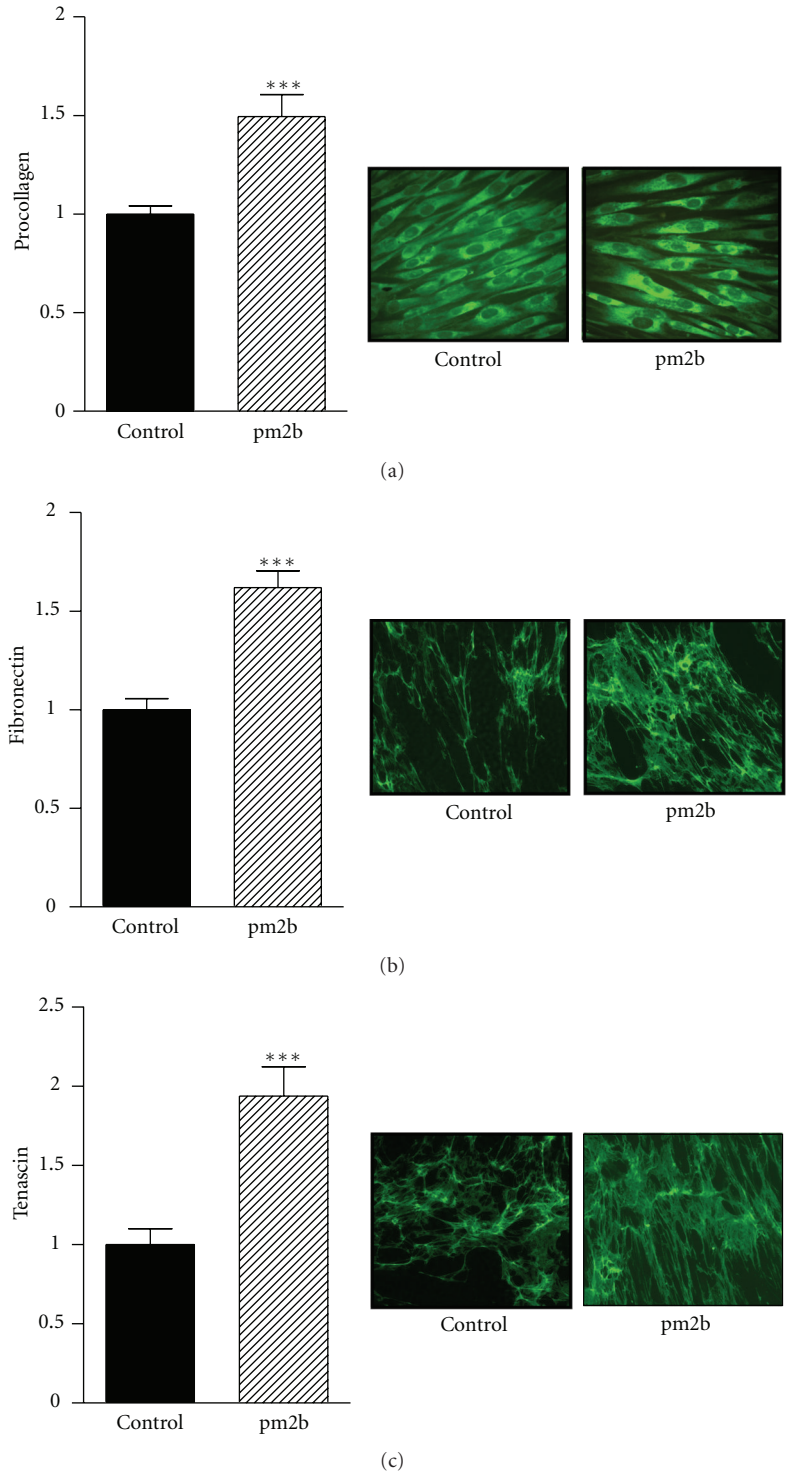

Figure 1: Extracellular matrix proteins in fibroblast culture. Procollagen (a), cellular fibronectin (b), and tenascin (c). Primary human fibroblasts were treated with pm2b (230 nM) and proteins were analyzed after 96 h by immunocytochemical staining (originally 400x). Data are representative images and expressed as mean ± SEM for triplicate measurements. $^{***}P < 0.001$ versus control.

Mice were euthanized prior to biopsies. All procedures were performed in compliance with the tenets of the Brazilian Society of Laboratory Animal Science (SBCAL/COBEA) and the institutional ethics committee.

2.7. Histological Analysis. Tissue sections of 3 μm thickness were stained with picrosirius red and examined under light microscopy (Carl Zeiss, Jena, Germany) coupled to Kontron 300 System Image Analyzer. Quantitative analysis of collagen

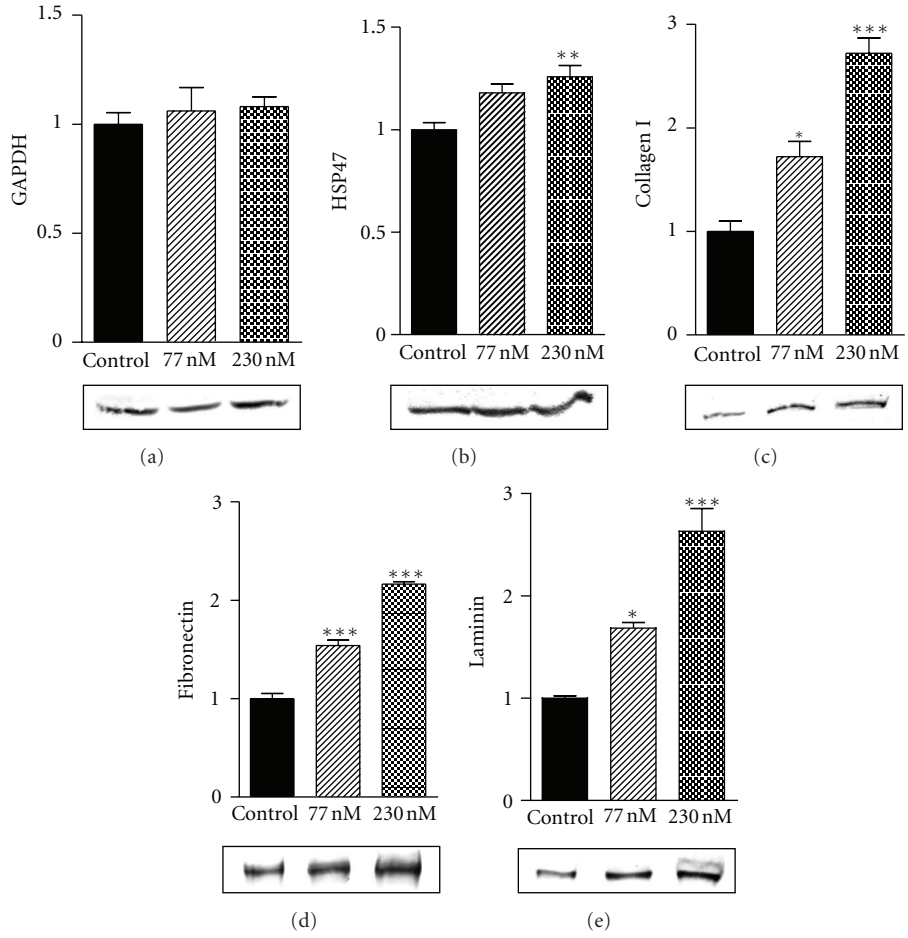

Figure 2: Extracellular matrix proteins in fibroblast lysate. Primary human fibroblasts were treated with pm2b (77 or 230 nM) and proteins were analyzed after 96 h by western blotting. Data are representative images and expressed as mean ± SEM for duplicate measurements. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ versus control.

was done from 10 microscopic fields at 200x magnification on flat sections of each biopsy through morphometric analysis and digital densitometry. Data were expressed as percentage of collagen staining to total area and in function of matched controls.

2.8. Calcium Mobilization Assay. Changes in free intracellular calcium concentration were measured by microfluorimetry using the FlexStation 3 (Molecular Devices, Sunnyvale, CA, USA) and FlexStation Calcium Assay Kit, following manufacturer's instructions. Fibroblasts were seeded at a density of 5×10^4 cells per well in black-well plates with clear bottom. Prior to experiments, cells were incubated for 1 h, at 37°C with the calcium kit reagent in serum-free medium. Before measurements, the following treatments were added: ATP (10 μM), thapsigargin (1 μM), and pm2b (80 or 230 nM, immediately and 1 h before). Both the direct effect of pm2b and also its influence on calcium mobilization by thapsigargin were investigated. The inhibitory effect of BAPTA (10 μM, 30 min before) was used as a positive interference control. Fluorescence was measured during 120 s, at 1.52 time intervals. Measurements were obtained as the difference between the peak intensity fluorescence and baseline.

2.9. Flow Cytometry Analysis. Cells were gently washed with PBS and detached with trypsin-EDTA. After addition of FBS 10%, the cells were harvested and washed twice with PBS. Pellets were resuspended in 4% paraformaldehyde and stored at 4°C. Antibody labeling was done according to manufacturer's instructions at room temperature. Prior to analysis, cells were permeated with 0.1% Triton X-100 for 30 min, incubated for 2 h with the respective antibodies for caspase 3, Bcl-2, Ki-67- MIB-1, cytochrome c (cyt c), IL-1β, CXCR1, CXCR2, IL-6R, or collagen-1 receptor ($\alpha 2\beta 1$), and then incubated with the secondary antibody Alexa Fluor 488 in the dark. Fluorescence-activated cell sorting (FACS) analysis was performed on a FACSCalibur flow cytometer, Becton Dickinson (San Jose, CA, USA). For each sample, at least 10,000 events were acquired and the data were evaluated using the Cell-Quest software.

2.10. Statistical Analysis. The difference among groups was analyzed by one-way or two-way analysis of variance

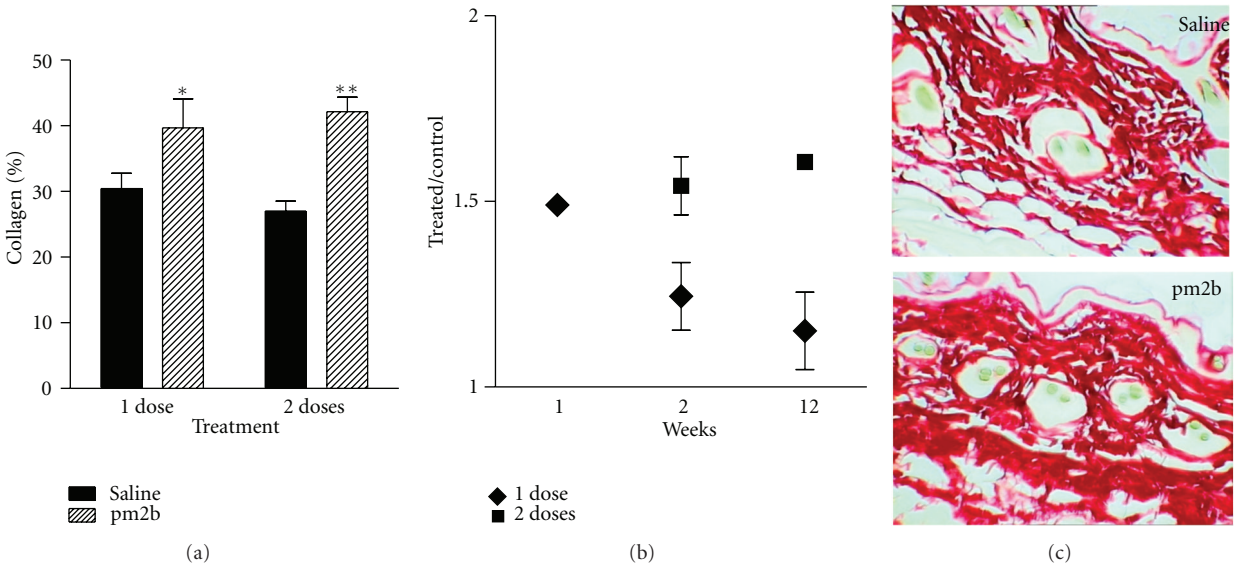

Figure 3: Peptide treatment increases collagen in the mice dermis. (a) Mean values of groups treated with a single dose (0.2 mL, 1.15 μM pm2b, i.d.) or repeated doses compared with controls ($n = 4$–6). (b) Matched observations of pm2b-treated and saline-treated sites (1 × 1 cm) in the same animals in successive intervals after treatment. (c) Picrosirius-red-stained sections (originally 340x). Data are representative images and expressed as mean ± SEM. $^{**}P < 0.01$, $^{*}P < 0.05$ versus control.

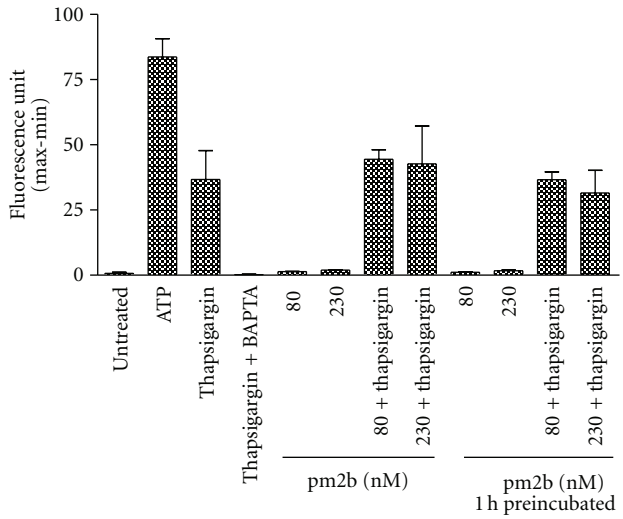

Figure 4: Calcium mobilization in fibroblasts. Fibroblasts were treated with pm2b at different concentrations immediately and 1 h before the test, and with thapsigargin (1 μM), BAPTA (10 μM, 30 min before), and ATP (10 μM). Data are expressed as mean ± SEM for duplicate measurements.

(ANOVA) and Student's t-test. Data are expressed as mean ± standard error (SEM) and representative images from each group. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Increased Production of ECM Proteins in Fibroblast Culture. To evaluate if pm2b was able to modulate fibroblast response, we assessed if the peptide could interfere with the production of ECM proteins by primary human fibroblasts in culture.

Immunofluorescence results showed that treatment with pm2b induced a significant increase of procollagen, fibronectin, and tenascin, as shown in Figure 1. In comparison to nontreated cultures, pm2b-treated fibroblasts showed almost onefold increase in tenascin (94%) and an increase in 49% of procollagen and 62% of fibronectin. Analysis of collagen type I, fibronectin, and laminin in the cell lysates by western blotting showed a significant increase in all these proteins in cultures treated with the peptide at 70 or 230 nM and a slight increase in HSP47 (Figure 2).

3.2. Increased Production of Collagen In Vivo. Since the peptide treatment induced a change in the content of ECM proteins in vitro, we assessed whether if pm2b was also able to increase the amount of collagen in vivo in the mice dermis (Figure 3). Interaction of pm2b treatment and the collagen content was statistically significant (two-way ANOVA, $P < 0.05$), either if it was lower than that observed in vitro. Treatment with a single dose induced a mean increase of local collagen fibrils in about 10%, while with two repeated doses the mean increase was 15% (Figure 3(a)). With a single dose, the higher difference to controls was observed 7 days after treatment. The ratio between treated area and control dropped along the time. Interestingly, in the group treated with 2 doses of pm2b, the collagen increase lasted for 3 months (Figure 3(b)).

3.3. Calcium Mobilization. Increase of intracellular calcium in fibroblasts was observed by using ATP or thapsigargin (Figure 4). As expected, pretreatment with BAPTA abolished

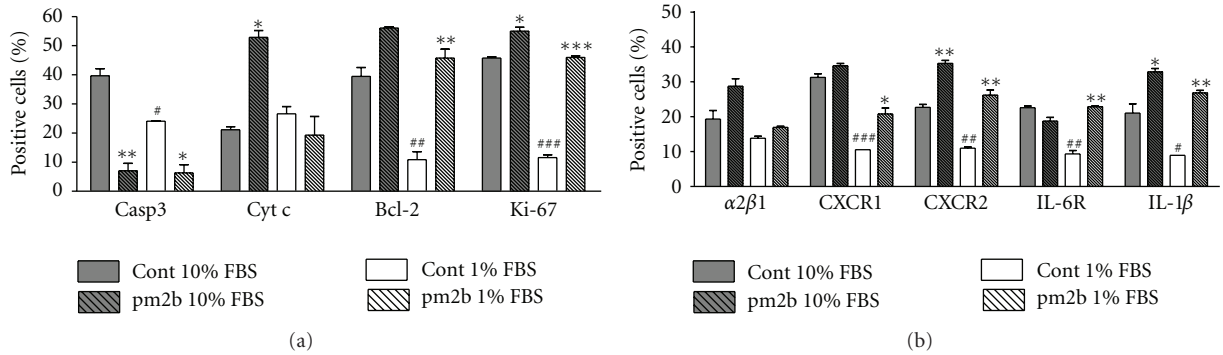

Figure 5: Differential expression of mediators involved in cell viability (a), cytokine and receptors (b). Fibroblasts were cultured with pm2b (230 nM) for 72 h with 10% or 1% FBS and analyzed by FACS. Data are expressed as mean ± SEM for triplicate measurements. $^{***}P < 0.01$, $^{**}P < 0.01$, $^{*}P < 0.05$ versus controls. $^{###}P < 0.01$, $^{##}P < 0.01$, $^{#}P < 0.05$ versus controls with 10% FBS.

the effect of thapsigargin. On the other hand, the peptide investigated showed no direct effect on intracellular calcium mobilization. In addition, it did not seem to interfere with the action of thapsigargin.

3.4. Modulation of Mediators Involved in Cell Viability and Inflammation. To investigate the mechanisms underlying fibroblast responses induced by pm2b, a set of mediators expressed by the cells in 1% and 10% FBS-supplemented medium were analyzed. Either treated or nontreated cultures showed different responses in these two conditions. pm2b promoted a synergistic modulation of mediators involved in apoptosis, antiapoptosis, and proliferation, resulting in a prosurvival response. As seen in Figure 5(a), pm2b suppressed caspase-3 and upregulated Bcl-2 and Ki-67. As shown in Figure 5(b), increases in CXCR1 and IL-6R were observed only with 1% FBS. On the other hand, cyt c was exclusively upregulated in 10% FBS-supplemented medium. In both conditions, there was a slight increase in the $\alpha 2\beta 1$expression, but not statistically significant. CXCR2 and IL-1β were markedly increased in both culture conditions.

4. Discussion

It is well known that morphogenesis and other physiological processes consist in a chain of events regulated by cross-talk signaling between ECM cells, receptors, and signaling factors [34]. However, lipocalin roles in these processes are not clearly understood. Regardless of many reports describing lipocalins as biomarkers of diseases [11] and others that correlate high lipocalin expression levels with stress conditions [35] and injury [16, 17], little is known about the biological activities of these multifunctional proteins and how they can modulate tissue and cell responses.

Lipocalins are classically recognized as carriers of lipophilic molecules. However, outgrowing data on the literature have indicated they are more than that. Some authors have suggested lipocalin motifs should play important roles in the structure pattern and functional properties of these proteins [31, 32, 36]. Bioinformatic analysis and peptide mapping indicated motif 2 is implicated in cell modulation and suggested it is a sequence signature with a role in cell survival [33]. However, its possible effects in fibroblasts were not known.

We have obtained a synthetic peptide with amino acid sequence based on the lipocalin motif 2, found in Lopap—an insect lipocalin from the *L. obliqua* caterpillar [37]. Lopap was previously shown to have a direct effect on endothelial cells increasing the surface expression of cell adhesion molecules, triggering IL-8 and nitric oxide release, and displaying antiapoptotic activity [36–40]. The peptide reproduced the effects observed with the whole protein, exhibiting an antiapoptotic activity in endothelial cells and neutrophils, which is dependent on nitric oxide synthase activity [33].

Herein, results demonstrate the peptide can modulate mediators favoring a prosurvival response, with suppression of caspase 3—a key proapoptotic enzyme, and up-regulation of the antiapoptotic protein Bcl-2, as well as the proliferation marker Ki-67. Other studies have also attributed to lipocalins roles in cell survival in different cell lineages [41–43], which support the hypothesis of a common property among lipocalins. This effect can be important for the lipocalin roles in several developmental and repairing processes, for protective response to stress, as well as for their possible involvement in many diseases.

Interestingly, pm2b treatment increased ECM proteins in vitro and in vivo. Fibroblasts are metabolically active cells which major function is the production of ECM components [1]. Our findings suggest for the first time a lipocalin role in ECM modulation. This finding brings new insights to understanding the involvement of lipocalins in the pathophysiology of diseases involving ECM deposition/remodeling defects. The difference in the amount of collagen increase induced by pm2b in vitro and in vivo may be due to collagen degradation by matrix metalloproteinases or either the peptide dose used and its stability in the tissue. Ki-67 is absent in resting cells and present during all phases of cell cycle, but otherwise its role in ribosomal RNA synthesis [44] may be related to the increase in synthesis activity observed in fibroblasts.

Fibroblast modulation by pm2b was shown to involve cytokine signaling favoring a proinflammatory response. Besides the previously reported modulation of IL-8 by Lopap [39], results showed the induction of the IL-8 receptors. Expression of the chemokine receptor CXCR1 was increased only in serum-deprived cultures, while expression of its paralog CXCR2 was increased no matter the assay condition. IL-8 and its receptors are known to have an autocrine role in cell survival [45]. There are also reports describing the involvement of IL-1 [46] and IL-6/IL-6R [47] in antiapoptotic responses. On the other hand, a local proinflammatory response can contribute for healing and regeneration [48]. However, the mechanisms by which Lopap and derived peptide trigger cell responses have to be investigated. As results show, it does not seem to involve changes in calcium transients.

To our knowledge, this is the first report of lipocalins modulating fibroblasts and ECM proteins, which could be directly implicated to their roles in morphogenesis and tissue homeostasis. The involvement of growth factors and cytokines in development and repairing process is well described [49]. However, lipocalins may be also considered as important players in these processes. Therefore, the mechanisms by which these proteins can trigger cell modulation have to be carefully investigated. Understanding the effects of these proteins can open perspectives for their use in prognosis and treatment of many dysfunctions involving wound healing, tissue remodeling, and cell death.

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Review Article

Towards Therapeutic Applications of Arthropod Venom K^+-Channel Blockers in CNS Neurologic Diseases Involving Memory Acquisition and Storage

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Potassium channels are the most heterogeneous and widely distributed group of ion channels and play important functions in all cells, in both normal and pathological mechanisms, including learning and memory processes. Being fundamental for many diverse physiological processes, K^+-channels are recognized as potential therapeutic targets in the treatment of several Central Nervous System (CNS) diseases, such as multiple sclerosis, Parkinson's and Alzheimer's diseases, schizophrenia, HIV-1-associated dementia, and epilepsy. Blockers of these channels are therefore potential candidates for the symptomatic treatment of these neuropathies, through their neurological effects. Venomous animals have evolved a wide set of toxins for prey capture and defense. These compounds, mainly peptides, act on various pharmacological targets, making them an innumerable source of ligands for answering experimental paradigms, as well as for therapeutic application. This paper provides an overview of CNS K^+-channels involved in memory acquisition and storage and aims at evaluating the use of highly selective K^+-channel blockers derived from arthropod venoms as potential therapeutic agents for CNS diseases involving learning and memory mechanisms.

1. Introduction

Many efforts have been made to understand the physiological mechanisms responsible for learning and memory. Due to their complexity, different approaches have been used to unlock them and various actors of these phenomena have been often revealed [1, 2]. In the last two decades, a new agent has gained the attention of the scientific community studying the processes of learning and memory: the potassium channels [3].

Potassium channels (KCNs) exhibit a great diversity (for review see [4, 5]). In mammals, nine and ten genes that encode channels for Na^+ and Ca^{2+} have been described, respectively. Nonetheless, for KCN they are 78 genes, at least [5]. In addition to this large number of genes, alternative splicing, RNA editing, posttranslational modifications, and channel formation of heteromeric assembly by the association of different principal subunits also contribute to the diversity of KCN [4]. These channels can be grouped into four families: voltage-gated channels (K_v), calcium-activated channels (K_{Ca}), inward-rectifiers channels (K_{ir}) and two tandem-pore channels (K_{2P}). Furthermore, these families have many subfamilies each containing several members: K_v have 12 subfamilies (K_v1-K_v12), K_{Ca}, 5 subfamilies ($K_{Ca}1$-$K_{Ca}5$), the K_{ir}, 7 subfamilies ($K_{ir}1$-$K_{ir}7$), and K_{2P}, 15 subfamilies ($K_{2P}1$-$K_{2P}7$, $K_{2P}9$,$K_{2P}10$, $K_{2P}12$, $K_{2P}13$ and $K_{2P}15$-$K_{2P}18$) [6–9].

The four families of KCNs are structurally related. K_v, K_{ir}, and K_{Ca} are transmembrane proteins formed by four α-subunits, whereas in K_{2P} there are only two. α-Subunits of all K_v, $K_{Ca}2$, and $K_{Ca}3$ have six transmembrane segments (6TM), while in $K_{Ca}1$, $K_{Ca}4$, and $K_{Ca}5$ are 7TM. All K_{ir} have α-subunits with 2TM and those of K_{2P} contain 4TM. When α-subunits join to form a channel, they may be identical and thus generating homodimeric or homotetrameric (homomultimeric) channels or may be different resulting

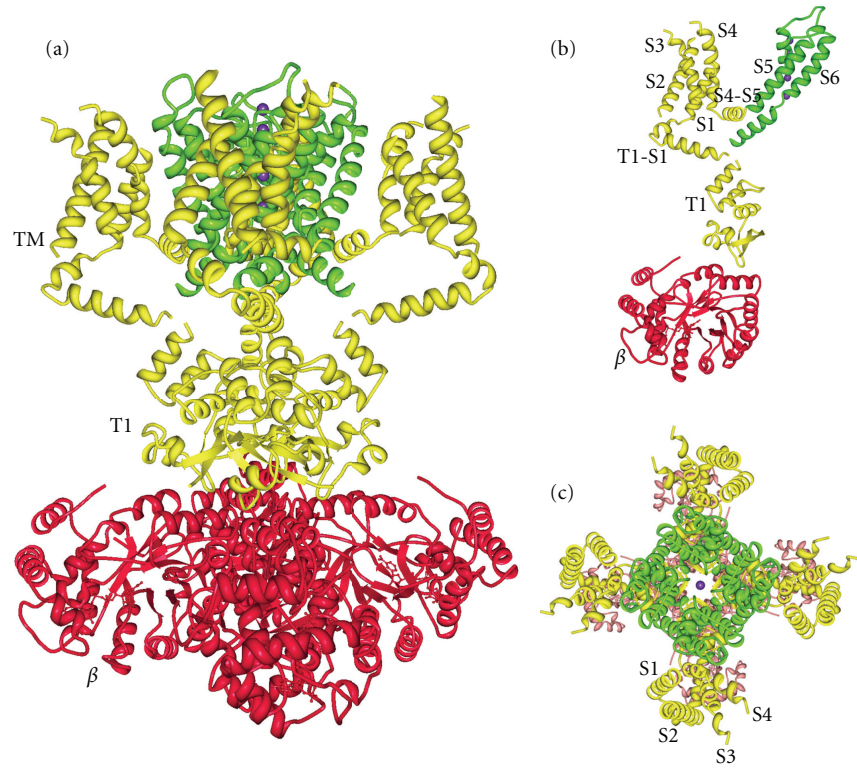

FIGURE 1: Views of the $K_v1.2$-β_2 subunit complex. (a) Side view of the $K_v1.2$-β_2 structure with the extracellular solution above and the intracellular solution below. Four subunits of the channel are colored in yellow (T1 domain and voltage sensor) and green (pore). β subunit tetramer is colored in red. TM indicates the integral membrane component of the complex. (b) Stereoview of a single subunit of the channel and β subunit viewed from the side. Labels correspond to six transmembrane helices (S1 to S6). (c) View of the $K_v1.2$-β_2 structure from the extracellular side of the membrane. S1–S4 helices from each subunit form four surrounding voltage-sensing domains, S5-S6 regions (green) from the four subunits shape a single pore domain. Purple spheres are potassium ions. Images were generated using Protein Workshop Viewer 3.9 [15] and Protein Data Bank accession ID 2A79 [14].

in heterodimeric or heterotetrameric (heteromultimeric) channels. Heteromultimeric channels are formed by the association of different α-subunits of the same subfamily. α-Subunits assembly of K_{2P} forms two pores and the in other KCNs there is one pore (for reviews, see [4, 5, 10–13]). The X-ray structure of mammalian $K_v1.2$ channel with the β_2 subunit (Figure 1) was reported by Long et al. [14]. In accordance with what is mentioned above, K_v channels are formed by four α-subunits that generate one pore (Figures 1(a) and 1(c)). Each $K_v1.2$ channel subunit contains six transmembrane segments, termed S1 to S6 (Figure 1(b)). The S5-S6 regions (Figures 1(a)–1(c), green) from the four subunits shape a single pore domain. The S1-S4 helices from each subunit form four surrounding voltage-sensing domains (Figures 1(b) and 1(c)).

KCNs have a significant influence on the neuron's activity, functioning of neuronal circuits, and brain plasticity. These channels regulate action potential firing patterns and control neurotransmitter release by constraining local membrane excitability and limiting Ca^{2+} influx (for review, see [16]). Furthermore, KCNs participate in the induction of synaptic plasticity by shape excitatory postsynaptic potentials (EPSPs) and enhance synaptic integration through their N-methyl-D-aspartate receptor (NMDAR), the cellular analogue for learning and memory (for review, see [3]). Therefore, KCNs may have an important role in cognition.

This possible involvement of KCN in cognitive processes is reinforced by its strong presence in Central Nervous System (CNS). These channels have a wide distribution in the brain of mammals. Several members of the K_v families [17–20], K_{Ca} [21–25], K_{ir} [26–29], and K_{2P} [30, 31], are found in the telencephalon, diencephalon, brainstem, and cerebellum of mammals.

The use of KCN blockers has aided to elucidate the function of these channels in the CNS. The tetraethylammonium (TEA) and 4-aminopyridine (4-AP) are traditional (classical) pharmacological blockers for KCN [32]. However, they are not specific, neither act on all types of KCN. TEA blocks different subtypes of K_v channels (except $K_v4.2$, $K_v4.3$, $K_v5.1$, $K_v7.1$ and all of the subfamilies K_v6, K_v8-K_v12), K_{Ca} (except subfamily $K_{Ca}2$ and $K_{Ca}3$), and subtypes $K_{ir}3.4$ and $K_{ir}7.1$, but has no effect on K_{2P} channels (for review see [6–9]). The 4-AP also operates on different subtypes of K_v channels (except $K_v3.4$, $K_v5.1$, and all K_v6-K_v12), $K_{ir}3.4$, and

$K_{ir}7.1$, but not on K_{Ca} channels, neither on the K_{2P} [6–9]. Therefore, the potential of these blockers to investigate the role of KCN on cognitive processes is limited.

Their diversity, distribution, and function suggest that the potassium channels could be involved in different cognitive processes, leading to a complex scenario that embarrass the understanding of the role of each K^+-channel subtype in these events. This challenge is even more complicated by the lack of drugs that act specifically on each type of KCN. One way to solve this problem has been the use of toxins isolated from invertebrates' venoms as K^+-channels blockers.

Many animal toxins, such as those isolated from spider, scorpion, and bee venoms, are specific blockers of potassium channels. While TEA operates in millimolar concentrations, scorpion toxins bind to and block K_v channels with pico- or nanomolar affinities [33]. Apamin, a toxin isolated from the bee venom, blocks $K_{Ca}2.2$ and $K_{Ca}2.3$ channels with affinity of the order of picomolar [34]. Therefore, animal venom toxins may be more useful as pharmacological tools than the classical blockers (TEA and 4-AP) as they act on K^+-channels with high potency and selectivity [35].

The aim of this paper is to review the knowledge accumulated on the importance of KCN to the processes of learning and memory. In addition, we present the contribution and potential use of toxins isolated from spiders, scorpions, and bees as pharmacological tools in this investigation. Finally, the participation of KCN in clinical conditions holding cognitive deficits and the possible use of toxins from animals as therapeutic agents are also considered.

2. Potassium Channels in Learning and Memory

The hippocampus has a great importance in learning and memory processes. This limbic structure holds a key in the consolidation of explicit memory. It receives information of events and transfers them to the neocortex where they are stored for a period (even weeks) and then gradually returned to specific regions of the cerebral cortex contributing to the formation of long-term memory [36].

The hippocampus expresses many types of KCN. As shown in Table 1, 39 different types of KCN belonging to the four families (K_v, K_{Ca}, K_{2P}, and K_{ir}) have been identified in this neural structure. Of these, the more expressed channels in the hippocampus appear to be the $K_v7.2$, $K_v7.3$, $K_{Ca}1.1$, $K_{ir}3.2$, and $K_{ir}3.3$, followed by $K_v1.1$, $K_v1.6$, $K_v3.1$, $K_v4.2$, $K_v10.1$, $K_{Ca}2.1$, $K_{Ca}2.2$, and $K_{ir}3.1$ (Table 1). What would be the role of this KCN diversity in the hippocampus?

Many experimental studies (Table 2) show that KCN may have a significant contribution in learning and memory processes. In these studies, the activity or expression of K^+ channels in the brain of rats and mice was altered by different strategies. The impact of this manipulation on the learning and memory was accessed by behavioral tests.

Ghelardini et al. [50] worked with mice subjected to passive avoidance test (Table 2). They found that intracerebroventricular (i.c.v.) administration of minoxidil, pinacidil, and cromakalim (KCN openers) produced amnesia. However, TEA, gliquidone and glibenclamide (KCN blockers) prevented this effect. These researchers also used toxins (apamin and charybdotoxin) whose results will be addressed later in the present paper.

Vick et al. [53] studied mice submitted to object recognition task, contextual fear-conditioning paradigm, and tone fear-conditioning paradigm (Table 2). Systemic 1-ethyl-2-benzimidazolinone (EBIO) and cyclohexyl-[2-(3,5-dimethylpyrazol-1-yl)-6-methyl-pyrimidin-4-yl]-amine (CyPPA), $K_{Ca}2$ channel activators, impaired the encoding, but not retrieval, of object memory in a spontaneous object recognition task. In addition, EBIO did not affect contextual or cued fear memory. They also tested apamin and this treatment will be discussed in Section 4.1.

Matthews and Disterhoft [54] observed rats submitted to trace eyeblink conditioning (Table 2). Intrahippocampal (CA1) injection of paxilline, a $K_{Ca}1.1$ channel blocker, resulted in slowed learning of the task.

Hammond et al. [55] produced transgenic mice that overexpress $K_{Ca}2.2$ subunits by 10-fold and tested them by Morris water maze, contextual fear-conditioning paradigm, and tone fear-conditioning paradigm on mice (Table 2). They found that this condition impaired learning in all tasks. The same results for these animals in contextual fear-conditioning paradigm were obtained for Stackman et al. [56].

Jacobsen et al. [57] tested doxycycline-induced conditional $K_{Ca}2.3$-deficient mice in five distinct learning and memory paradigms: passive-avoidance test, Morris water maze test, object recognition task, Y-maze test, and five-trial inhibitory avoidance test. Impairment was only observed in the last two tasks (with no effect on the others).

Deng et al. [58] worked with rats subjected to Morris water maze test (Table 2). They infused small interfering RNA (siRNA) in the entorhinal cortex (EC) to knock down $K_{2p}10.1$ channels. Baclofen, a specific γ-aminobutyric acid (GABAB) receptor agonist, was also applied into EC. The treatment of rats with siRNA abolished baclofen-induced inhibition of spatial learning. When administered alone, siRNA tended to improve the learning ability of rats.

Wickaman et al. [46] employed mice lacking a functional $K_{ir}3.4$ gene and submitted them to passive-avoidance test and Morris water maze test (Table 2). These mice performed similarly to wild-type controls in the first task, however, exhibited impaired performance in latter.

Betourne et al. [48] worked with wild-type and $K_{ir}6.2$ knockout mice submitted to Morris water maze test, contextual fear-conditioning paradigm, and tone fear-conditioning paradigm (Table 2). In wild-type mice, intra-hippocampal (CA3) injection of diazoxide ($K_{ir}6.2$ opener) impaired contextual memory. This effect was reversed by co-injecting tolbutamide ($K_{ir}6.2$ blocker). The $K_{ir}6.2$ knockout mice presented impairment of contextual and tone memories and slightly impaired performance in Morris water maze (special memory).

It has been described that $K_v1.1$ channels contribute to the processes of learning and memory. Meiri et al. [51] were able to inhibit the expression of $K_v1.1$ in the hippocampus,

Table 1: Distribution of different types of K^+ channels in hippocampus.

Channels	Hippocampus			Employed technique	Ref.
	CA1	CA3	DG		
$K_v1.1$	++	+++	+++	ISH, IMH, IMC, and CIMP in hippocampus or brain of rat, mouse, or gerbil.	[37–39]
$K_v1.2$	+	+	++		
$K_v1.3$	−	−	−	IMH in gerbil hippocampus.	[39]
$K_v1.4$	++	++	++	ISH, IMH, and IMC in hippocampus or brain of rat, mouse or gerbil.	[20, 38, 39]
$K_v1.5$	+	+	−	IMH or single-cell RT-PCR in gerbil or rat hippocampus.	[39, 40]
$K_v1.6$	++	+++	+++	IMH in gerbil hippocampus.	[39]
$K_v2.1$	++	++		Single-cell RT-PCR in rat hippocampus.	[17]
$K_v3.1$	++	+++	+++	Northern blot analysis and ISH in rat brain.	[17]
$K_v3.2$	+++	++	−		
$K_v3.3$	+	+	++		
$K_v3.4$	−	−	++		
$K_v4.1$	+	+	++	ISH in rat brain.	[18]
$K_v4.2$	+++	++	+++	ISH or IMH in rat or mouse brain.	[18, 41, 42]
$K_v4.3$	+	++	+++	ISH in rat brain.	[18, 42]
$K_v7.2$	+++	+++	+++	ISH and IMH in rat brain	
$K_v7.3$	+++	+++	+++		
$K_v10.1$	++	+++	++	ISH, real time PCR, or IMH in rat brain.	[19, 43]
$K_v10.2$	−	−	−	ISH and IMH in rat brain.	[19]
$K_v11.1$	++	−	−		
$K_v11.2$	−	−	−		
$K_v11.3$	+++	−	−		
$K_v12.1$	+	−	+		
$K_v12.2$	++	−	++		
$K_{Ca}1.1$	+++	+++	+++	ISH, WB analysis, IMH, IMF, IMC, or RLB in mouse or rat brain.	[21, 22, 44]
$K_{Ca}2.1$	++	+++	++	ISH, IB analysis, IMH, or RLB in rat brain.	[23, 24]
$K_{Ca}2.2$	+++	+++	+	ISH, IB analysis, IMH, or RLB in rat brain.	[23–25]
$K_{Ca}2.3$	+	++	+		
$K_{2p}1.1$	−	++	++	ISH in rat and mouse brain.	[31]
$K_{2p}2.1$	++	+	+++	ISH, WB analysis, IMH, IMF, or IMC in rat or mouse brain.	
$K_{2p}3.1$	++	++	++	ISH in rat and mouse brain.	[31]
$K_{2p}4.1$	++	+++	+		
$K_{2p}9.1$	++	++	+++		
$K_{2p}10.1$	−	++	+		
$K_{ir}2.1$	+	+	+++	ISH or IMH in mouse or rat brain.	[26–28]
$K_{ir}2.2$	++	+	++	ISH in mouse or rat brain.	[26, 27]
$K_{ir}2.3$	++	+	+++		
$K_{ir}3.1$	+++	++	+++	ISH or IMH in rat brain.	[27, 28]
$K_{ir}3.2$	+++	+++	+++	ISH or IMH in rat brain.	[27, 45]
$K_{ir}3.3$	+++	+++	+++	ISH in rat brain	[27]
$K_{ir}3.4$	+	++	+	WB analysis, IMH or ISH in rat or mouse brain.	[27, 45–47]
$K_{ir}6.2$	++	++	++	ISH, IMH, or IMF in rat or mouse brain.	[29, 48, 49]

The symbols indicate signal intensity as follows: − (not detected); + (weak); ++ (moderate); +++ (high); CA1, CA3, and DG (Dentate gyrus) are regions of hippocampal formation; Ref.: reference; RT-PCR means reverse transcription polymerase chain reaction; ISH is used for *in situ* hybridization; IMH for immunohistochemistry; IMC for immunocytochemistry; CIMP for coimmunoprecipitation; IMF for immunofluorescence; WB for western blot; IB for immune blot; RLB for radioligand binding.

Table 2: K^+ channels manipulations and their effects on experimental behavioral models for learning and memory.

Channels	Technique	Effect on channel	Behavioral test	Result	Ref.
K_v, K_{Ca}, and K_{ir}	icv of minoxidil, pinacidil, TEA, glibenclamine, gliquidone, and cromakalim.	TEA blocks Kv and K_{Ca}. Gliquidone and glibenclamide block K_{ir}. Minoxidil, pinacidil and cromakalim open K_{ir}	PAT in mice	Minoxidil, pinacidil and cromakalim: (−) TEA, glibenclamine, gliquidone: (+)	[50]
K_v1.1	icv of antisense oligodeoxyribonucleotide to K_v1.1 mRNA.	Inhibition of channel expression	PAT in mice	(−)	[51]
			MWM in rats	(−)	
K_v12.2	K_v12.2 (BEC1) knockout mice	Inhibition of channel expression	MWM	(+)	[52]
			Y-maze test	(+)	
			WFT	(+)	
	K_v12.2 OVER mice	Overexpression of channel in the forebrain	MWM	(−)	
			Y-maze test	(−)	
			WFT	(−)	
K_{Ca}2	Systemic Infusion of EBIO or CyPPA.	EBIO activates SK channels. CyPPA activates K_{Ca}2.2 (SK2)/ K_{Ca}2.3 (SK3) subunits over K_{Ca}2.1 (SK1), and is more potent than EBIO.	ORT in mice	EBIO: (−) CyPPA: (−)	[53]
			Contextual FCP in mice	EBIO: (0) CyPPA: (NT)	
			Tone FCP in mice	EBIO: (0) CyPPA: (NT)	
K_{Ca}1.1	ih (CA1) of paxilline	Paxilline blocks the channel.	Trace eyeblink in rats	(−)	[54]

Table 2: Continued.

Channels	Technique	Effect on channel	Behavioral test	Result	Ref.
$K_{Ca}2.2$	SK2-OVER mice	Overexpression of $K_{Ca}2.2$ (SK2) protein and $K_{Ca}2.2$ mRNA	MWM Contextual FCP Tone FCP	(−) (−) (−)	[55]
$K_{Ca}2.2$	SK2-OVER mice	Overexpression of $K_{Ca}2.2$ (SK2) protein and $K_{Ca}2.2$ mRNA	Contextual FCP	(−)	[56]
$K_{Ca}2.3$	Doxycycline-induced conditional SK3 channel deficient (T/T) mice	Inhibition of channel expression	PAT MWM ORT Y-maze test Five-trial inhibitory avoidance test	(0) (0) (0) (−) (−)	[57]
$K_{2P}10.1$	Infusion of siRNA in the EC.	Knock down $K_{2p}10.1$ channels in the EC.	MWM in rats	(+)	[58]
$K_{ir}3.4$	$K_{ir}3.4$ (GIRK4) knockout mice	Inhibition of channel expression	PAT MWM	(0) (−)	[51]
$K_{ir}6.2$	ih (CA3) of diazoxide, or tolbutamide, or both.	Diazoxide opens the channel. Tolbutamide blocks the channel.	Contextual FCP in mice Tone FCP in mice	Diazoxide: (−) Tolbutamide: (0) Both: (0) Diazoxide, tolbutamide or both: (0)	[48]
	$K_{ir}6.2$ knockout mice	Inhibition of channel expression	Contextual FCP Tone FCP MWM	(−) (−) (0/−)	

(−): impairment; (+): improved; (0): neutral; (0/−): slight impairment; (NT): not tested; Ref.: reference; icv: intracerebroventricular injection; ih: intra-hippocampal injection; EC: entorhinal cortex; OVER: overexpressing; EBIO: 1-ethyl-2-benzimidazolinone; CyPPA: Cyclohexyl-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-pyrimidin-4-yl]-amine; siRNA: small interfering RNA; PAT is used for passive-avoidance test; MWM for Morris water maze test; WFT for Water-finding task; ORT for object recognition task; FCP for fear-conditioning paradigm.

blocking the translation of the mRNA of these channels. They found that this procedure worsened the passive avoidance in mice and spatial memory in rats (Table 2). Kourrich et al. [59], working with odor-discrimination tasks in rats, showed that levels of mRNA expression of $K_v1.1$ channels in the hippocampus were positively correlated with associative learning.

On the other hand, $K_v2.1$ channels appear to interfere negatively with learning and memory. Zhong et al. [40] suggested that memory deficits induced by scopolamine in rats may result from exacerbation of potassium currents in hippocampal pyramidal neurons as a consequence of increased mRNA expression of $K_v2.1$ channels.

It has been shown that $K_v12.2$ (BEC1) channels have preferential distribution in the forebrain, including the hippocampal and cortical regions [19, 60, 61]. The use of $K_v12.2$ knockout and overexpression (OVER) mice made possible to verify that this KCN is negatively involved in cognitive function, since the $K_v12.2$ knockout mice performed behavioral tasks related to working memory, reference memory, and attention better than their wild-type animals. In the OVER mice, on the other hand, the performance of those tasks was impaired [52] (Table 2).

Taken together, these studies strongly suggest that the brain KCNs and their modulation play an important role in the regulation of memory processes. Some hippocampal K_v (K_v1, K_v2 and K_v12), K_{Ca} ($K_{Ca}1$ e $K_{Ca}2$), and K_{ir} ($K_{ir}3$ e $K_{ir}6$) channels seem to be particularly relevant. On this basis, the KCN blockers could be useful in investigation and the treatment of cognitive deficits.

3. Arthropod K^+ Channels Toxins

Arthropod venoms constitute a rich source of peptidyl KCN inhibitors (KTxs). There are two classes of inhibitory peptides based on their mechanism of action: (1) KCN blockers which bind to the outer vestibule and then blocking the ion conductance by the pore occlusion; (2) KCN gating modifiers which shift the channel opening to more positive potentials. Scorpion KCN blockers (scorpions KTxs), exemplified by charybdotoxin (ChTX) [62], and the peptide tertiapin, isolated from bee venom [63], act as pore blockers, while spider KTxs, such as SGTx1 [64], act as a gating modifier. In turn, apamin, another toxin from bee venom, possibly acts as a pore blocker, although residues of the extracellular S3/S4 loop of the $K_{Ca}2$ (SK) channels also affect the apamin binding [65]. According to Lamy et al. [66], based on differences in binding affinity and potency of the blockage, apamin does not behave as a classical pore blocker, and probably the blocking effect occurs by an allosteric mechanism.

These KTxs show different arrangement of their three-dimensional (3D) structures. The folding types earlier found are $\alpha\alpha$, $\alpha\beta\beta$, and $\beta\alpha\beta\beta$ [67–69]. Despite the conformation differences, most of these peptides have common residues which promote the binding with the potassium-channel vestibule, such as a lysine residue distant from an aromatic residue for 6.6 ± 1.0 Å [70].

Arthropod toxins have been used as pharmacological tools to better understand the role of ion channels, as most of them act in a high specific and potent way. Some of these toxins constitute unique blockers of certain ion channels, such as ergtoxin-1 (*Centruroides noxius*) [71], and BeKm-1 (*Mesobuthus eupeus*) [72] for $K_v11.1$ (HERG), psalmotoxin (*Psalmopoeus cambridgei*) for ASIC1a channel [73], tertiapin-Q (*Apis mellifera*) for $K_{ir}3.1$ (GIRK) [63], and Lq2 (*Leiurus quinquestriatus hebraeus*) for $K_{ir}1.1$ (ROMK1) [74].

The scorpion KTxs are formed by 20–95 amino acid residues stabilized by two, three, or four disulfide bonds, making this structure relatively stable. The scorpion KTxs were originally classified into three families named α, β, and γ [75], all of them have the highly conserved secondary structural arrangement α/β stabilized by cysteines (CSα/β). More recently, scorpion KTxs presenting a different structural arrangement, with only two α-helices stabilized by two disulfide bonds, CSα/α, were described, and these peptides were named κ-KTxs [76–78]. Among the almost 190 scorpion KTxs described until now, the α-KTx family, the largest one, contains more than 130 peptides thus far, classified in 20 subfamilies, based on their amino acid homology [75, 79].

Toxins from spider venom can play an important and complementary role in investigation of KCN cognitive function. Unlike most animal toxins obtained from snakes, bees, scorpions and sea anemones venom, which block mainly K_v1 and K_v3 channels, peptide toxins from spiders target K_v2 and K_v4 channels, which are expressed in the CNS and cardiovascular system of mammals (for review see [80]). Moreover, the venom spiders belonging to the Theraphosidae family represent a plentiful source of peptides that modify the gating of K_v channels [81]. Hanatoxin and seemingly others tarantula toxins shift channel opening to more depolarized voltages [81, 82] by stabilizing the resting conformation of the voltage sensor [83]. It has been suggested that these peptides interact with the voltage-sensor paddle within the lipid membrane [84–86].

The bee venom is composed of several classes of peptides, as well as enzymes and biogenic amines. Among the peptides, it can be emphasized the presence of the melittin [87], apamin [88], tertiapin [89], and mast cell degranulating peptide [90]. Certainly, in relation to neurotoxins, the apamin has greatly excelled, since this peptide has been considered a good pharmacological tool and it has provided important information in respect of the functioning of K^+-channels [91].

4. Arthropod KTxs in Learning and Memory

As already mentioned above, KCN classical blockers are not specific and cannot act at all KCNs. So these blockers have a major limitation as tools for studying the role of KCN in the CNS. An alternative to this obstacle is the use of KTxs from arthropods venom. In this section we report studies using these toxins to access the mechanisms of learning and memory in animals submitted to behavioral tests. Table 3 presents a summary of these works. Analyzing this table, we can see that the apamin is the most used KTx in tasks of learning and memory, as detailed below.

Table 3: Assembled data on the effect of the bee and scorpion venom KTxs on the performance of animals in behavioral tests of learning and memory.

	Species	Toxin	KCN target	Behavioral test	Result	Ref.
Scorpion	*Androctonus mauretanicus mauretanicus*	Kaliotoxin	$K_v1.1$ and $K_v1.3$ blocker	Olfactory discrimination task in rats	Improvement	[92]
	Buthus tasmulus	Iberiotoxin (IbTx)	$K_{Ca}1.1$ blocker	Passive avoidance test in chicks	Impaired retention	[93]
	Leiurus quinquestriatus hebraeus	Charybdotoxin	$K_v1.3$ and $K_{Ca}1.1$ blocker	Passive avoidance test in mice	Improvement	[50]
		Lei-Dab7	$K_{Ca}2.2$ blocker	Radial arm maze in rat	No effect	[25]
Bee	*Apis mellifera*	Apamin	$K_{Ca}2.2$ and $K_{Ca}2.3$ blocker	Bar-pressing response in appetitively motivated mice	Improvement	[94]
				Object recognition task in rats	Improvement	[95]
				Habituation task in rats	Improvement	[96]
				Passive avoidance test in rats or mice	No effect	[96, 97]
				Passive avoidance test in mice	Improvement	[50, 98]
				Morris water maze in mice	Improvement	[97, 98]
				Morris water maze in rats	No effect	[98]
				Y-maze test in mice	No effect	[97]
				Radial arm maze test in mice or rats	Improvement	[25, 99]
				Visual discrimination in rats	No effect	[98]
				Olfactory discrimination task in rats	Improvement	[100]
				Olfactory associative task in rats	Improvement	[101]
				Tone fear-conditioning paradigm in mice	No effect	[53]
				T-maze test in rats	Improvement	[102]
				Passive avoidance task in chicks	Impaired retention	[103]

Ref.: reference.

4.1. Apamin. Apamin is a small peptide that corresponds to less than 2% of bee venom dry weight. Its amino acid sequence was described independently by two groups, revealing completely its primary structure [104]. Like many other peptide neurotoxins, apamin has high cysteine content and a high basicity, but apamin is different from most peptide toxins in its unusual ability to cross the blood brain barrier and act on the Central Nervous System [105]. This peptide is a polypeptide of 18 amino acids having a molecular weight of 2039 Da, with two disulfide bridges connecting position 1 with 11, and position 3 with 15 [106]. According to Vincent et al. [107], the most important part of the apamin sequence for neurotoxic activity appears to be the C-terminal region containing the two arginine residues, given that chemical modification of Argl3 and Arg14 eliminates toxicity (DL_{50} in mice). Assays with autoradiography of binding sites for apamin revealed that it binds preferentially to the hippocampus, to the habenular nucleus, and to the nucleus medialis septi [108].

Apamin is an antagonist of all three subtypes of small conductance Ca^{2+}-activated K^+-channel, $K_{Ca}2$ channels. However, this KTx showed subtype-specific affinity demonstrated by values of half maximal inhibition (IC_{50}) and, dissociation constant (Kd) for $K_{Ca}2.1$ (SK1), $K_{Ca}2.2$ (SK2), and $K_{Ca}2.3$ (SK3) (IC_{50} = 704pM, 27pM, 4 nM and Kd 390pM, 4pM, 11pM, resp.) [34]. As a consequence of pharmacological blockage, apamin has been considered as a useful tool to investigate the physiological mechanisms involved in higher brain functions, especially cognitive processes or in the control of mood [98, 109].

This bee KTx facilitates spatial and nonspatial learning and improves memory performance in laboratory rodents, accelerating the acquisition of bar-pressing response in appetitively motivated mice [94]. In that report, when apamin was injected (dose 0.2 mg/kg, i.p.) 30 min before the acquisition session, it accelerated lever-press learning and the performance in a retention session 24 h later in BALB/mice. Results showed that immediate administration improved the retention of the lever-press task one day later [94]. Additionally, administration of the apamin in the acquisition of the lever-press task produced a stronger increase in early gene expression, *c-fos* and *c-jus*, in the CA1, CA3, and dentate gyrus as compared to trained saline-injected mice [110]. The similar pattern of immediate genes has been observed in the initial activation of neurons during the memory process [99].

Moreover, Deschaux et al. [95] showed that injection of apamin (0.4 mg/kg i.p.) before the training improved learning in an object recognition task in rats. Emphasizing, they found that rats injected with apamin before the first exploration session spent more time in exploring the new object than the familiar object at the second trial, when it took place 24 h after the first trial. Injection of apamin just after the first trial or before the second trial did not modify the difference in exploration time between the new and the familiar object. These results suggest that apamin could improve learning, but not consolidation or restitution of the information, in an object recognition task [95].

Also in 1997, Deschaux and Bizot [96] reported that, in the habituation task, apamin (0.4 mg/kg, i.p) decreased activity (distance travelled and rearing) on the restitution sessions only when it was injected before acquisition sessions, but not when injection took place just after the acquisition session or before the restitution session. In addition, the authors showed that in the passive avoidance test, apamin did not alter performance whenever the time of administration. According to Deschaux and Bizot [96], blockage of the apamin sensitive K_{Ca} channels improved the acquisition in nonstressful task, but not in a stressful situation in rats.

In the water maze spatial navigation, apamin (0.2 and 0.06 mg/kg, i.p) administered 30 min before daily training improved the acquisition and reversal learning of septal-lesioned mice, but it did not improve learning and memory in a spontaneous alternation task in a Y-maze and in a passive avoidance task, and did not affect learning and memory in any of three tasks when intact mice were used as subjects [97]. Interestingly, apamin dose dependently (0.2–0.06 mg/Kg, i.p) reversed the lesion-induced defect in the radial arm maze and in the water maze [99]. These results corroborated that blockage of K_{Ca} channels can alleviate the spatial reference memory and working memory defect induced by a damaged septohippocampal axis [99].

Van Der Staay et al. [98] make a comprehensive study using series of cognition tests with mice and rats from different strains. They used the standard version and a modified version of the Morris water escape task, the passive and active avoidance tasks, and the operant tasks in the Skinner box as cognitive tests, and the rat forced swimming test and the open-field test after cocaine administration as noncognitive tests. Results showed that apamin appeared to improve the cognitive performance of mice in two versions of the Morris water escape task and the passive avoidance task. However, inconsistence evidence was identified in rats. Moreover, apamin affected the general behavior of rats in the visual discrimination task, in the forced swimming test, and in the open field after cocaine administration [98].

In 2001, it was tested the effect of an i.c.v apamin (0.3 ng) injection on an olfactory associative task. Apamin did not modify the learning of the procedure side of the task or the learning of the odor-reward association. To specifically test reference memory, the rats were trained on a new odor-association problem using the same procedure (acquisition session), and they were tested for memory retention 24 h later. Apamin injected before or after the acquisition session improved retention of the valence of a new odor pair. Thus, the results indicate that the blockage of apamin-sensitive $K_{Ca}2$ channels facilitates reference memory [100].

In order to investigate the effect of potassium channel subtypes on rats learning and memory, Mpari et al. [101] compare the effects of two blockers, apamin (0.3 ng) and lei-Dab7 (3 ng), a modified scorpion KTx that selectively blocks $K_{Ca}2.2$ [111]. The results indicated that the blockage of $K_{Ca}2.2$ and $K_{Ca}2.3$ channels by apamin facilitates consolidation on new odor associations, using olfactory associative task in rats. However, lei-Dab7 remains without effect suggesting an involvement of $K_{Ca}2.3$ channels for the integration of synaptic signaling and plasticity modulation involved in learning and memory processes [101].

In the same way, in a spatial radial-arm maze task with rats, it was shown that lei-Dab7 did not modify attention or memory. However, apamin (specific to $K_{Ca}2.2$ and $K_{Ca}2.3$ channels) improved reference memory and accelerated strategy changes from egocentric to allocentric. These results reinforce that $K_{Ca}2.3$ blockage improves memory in rats [25].

Apamin is also able to facilitate the encoding of contextual fear memory during the limited 1 conditioned stimulus-unconditioned stimulus pairing protocol. It was shown that mice treated with apamin exhibited significantly greater freezing during the context test than did the saline-treated control [53].

More recently, the role of K_{Ca} in memory formation was explored in chicks trained on a single-trial discrimination avoidance task. Blockage of $K_{Ca}2$ channels using apamin (1 nM, 0.02 ng/hem, i.c.) impaired long-term memory retention when administered between 10 min before, and 30 min after training [103].

Blockage of $K_{Ca}2$ channels in the prefrontal cortex (PFC) by apamin also improves working memory performance [102] (Table 3). In prefrontal, visual, and somatosensory cortical pyramidal neurons, $K_{Ca}2$ channels mediate a hyperpolarization following calcium-induced calcium release, triggered by activation of muscarinic [112, 113] or glutamate receptors [114]. The PFC is central for working memory, the aptitude to internally symbolize information without an external input, being fundamental for establishing conceptual thinking, and for language, for example. As $K_{Ca}2$ channels play an important role in diminishing the excitability during synaptic transmission in the medial prefrontal cortex (mPFC), their blockage could potentiate synaptic transmission optimizing activity within the mPFC network [115, 116].

All these studies suggest that $K_{Ca}2$ channels are involved in memory processes but only when the task does not implicate a spatial strategy or a stressful situation. Since apamin is effective in some of these examples, but fails to have an effect in others, it appears that apamin-sensitive channels affect only certain circuitries involved in memory processing. Van Der Staay et al. [98] discuss the use of apamin as a tool to study the role of potassium channels in learning and memory. They do not consider apamin a good tool, despite its high selectivity, because the peptide has a very narrow therapeutic window, since there is an apparent overlap between the doses that enhance cognition with those causing side effects. Even so, studies using apamin are of great importance for the understanding of these channels and their influence on processes of learning and memory.

4.2. Charybdotoxin. Charybdotoxin (α-KTx 1.1, ChTX-Lq1, or ChTx-a), isolated from *Leiurus quinquestriatus hebraeus* (Yellow scorpion) [117], is a potent selective inhibitor of high (large or big) conductance Ca^{2+}-activated potassium channels ($K_{Ca}1.1$, BK, or maxi-K), as well as a $K_v1.3$ channel [62]. In an autoradiographic study of rat brain it was demonstrated high levels of [^{125}I]-charbydotoxin in white matter regions such as the lateral olfactory tract and fasciculus retroflexus, as well as in gray matter-containing regions such as the zona incerta, medial geniculate, and superior colliculus [118].

Using a [^{14}C]-2-deoxyglucose autoradiographic technique, it was shown that i.c.v. administration of charybdotoxin produced effect on glucose utilization in 21 brain regions predominantly limited to the hippocampus, limbic and motor structures, indicating that glucose utilization was altered within three pathways implicated within learning and memory processes, the septohippocampal pathway, Schaffer collaterals within the hippocampus, and the Papez circuit. These results suggested the possibility that handling of particular subtypes of K_v1 channels by specific scorpion toxins in the hippocampus and related structures could alter cognitive processes without provoking large-scale changes in neural activity throughout the brain [119].

Ghelardini et al. [50] showed that the i.c.v. administration of charybdotoxin, 20 min before the training session in the mouse passive avoidance test, prevented the amnesia induced by potassium-channel openers (minoxidil and pinacidil). The amnesia in mice was produced by opening the K_{ATP} potassium channels, and it was reversed by blocking K_{ATP} potassium channels. Since the prevention of the amnesia induced by K_{ATP} potassium channel openers in the mouse passive-avoidance test is also obtained by blocking voltage-gated and calcium-activated channels, it is plausible to consider that more than one type of KCN appears to be involved in cognitive processes. It is worth mentioning that KCN blockers used by Ghelardini et al. [50] did not improve cognitive abilities when given alone, contrasting with the findings showing that apamin enhances memory in an object recognition task [95]. In fact, apamin, as it blocks K_{Ca} channels, affects learning rather than memory, an effect that is noticeable only in an object recognition task and that was observed after an interval of 24 h, when the control animals were not able to remember the exploration of the objects presented in the first session anymore [50].

4.3. Kaliotoxin. Kaliotoxin is a specific inhibitor of $K_v1.1$ and $K_v1.3$ isolated from the scorpion *Androctonus mauretanicus mauretanicus* [120]. The involvement of $K_v1.1$ and $K_v1.3$ in learning and memory processes was studied by using kaliotoxin in rats submitted to olfactory associative learning. Kaliotoxin (10 ng) improved learning but not information consolidation in the odor-reward training and increased the long-term retrieval of an odor-reward association tested by a reversal test 1 month after the odor-reward training. The reference memory was also tested by successive odor-pair training. When kaliotoxin was injected before the acquisition or retention session it improved performance. Nevertheless, when kaliotoxin was injected immediately after acquisition no effect was observed, suggesting that the blockage of $K_v1.1$ or $K_v1.3$ channels by kaliotoxin facilitates cognitive processes as learning, in particular in a reference representation [92].

4.4. Iberiotoxin (IbTx). IbTx, a toxin isolated from venom of the scorpion *Mesobuthus tamulus*, is a selective inhibitor of $K_{Ca}1.1$ channels [121, 122]. These channels have been identified in brain regions that are related to cognition

such as the hippocampus [123], and they have also been implicated in memory processing.

In rabbits, it has been shown a strong relationship between classical conditioning and membrane excitability in Purkinje cells which persisted for at least 1 month [124]. In slices of cerebellar lobule, the administration of IbTX mimics the increases in membrane excitability related to conditioning, suggesting that classical conditioning is dependent upon the inhibition of $K_{Ca}1.1$ channels [124].

In chicks submitted to passive avoidance task, Edwards and Rickard [93] showed a transient retention loss (40–70 min after training) associated with the central administration of 50 nM IbTX immediately after training.

4.5. Spider KTxs. Despite K_v2 channels possibly contributing negatively to the mechanisms of learning and memory and spider venom being a rich source of gating modifiers of K_v2 channels, no evaluation of the use of the spider KTxs as probes in cognitive processes was carried out so far. Only one study [125] tested the effect of a spider toxin in mice submitted to behavioral tests of memory, but this toxin was not a KTx.

5. Potassium Channels and Clinical Conditions Holding Cognitive Impairment

The studies presented above suggest that, at least in part, expression modification and conductance modulation of potassium channels are related to learning and memory processes. In some neurological dysfunctions, the relationship between KCN and cognitive aspects is also evident.

5.1. Limbic Encephalitis. Limbic encephalitis is characterized by disorientation, agitation, anxiety, depression, irritability, personality change, acute confusional state, hallucinations, complex partial and secondary generalized seizures, and, mainly, by impairment of short-term memory. Limbic encephalitis may have paraneoplastic or autoimmune origin (for reviews see [126, 127]).

Some cases of limbic encephalitis reinforce that KCNs are involved in learning and memory processes. Patients with this clinical condition and high plasma concentration (as reflected in the cerebrospinal fluid) of antibodies against voltage-dependent potassium channels (anti-K_v) had severe memory impairment. This cognitive function has a neuropsychological improvement after reducing serum levels of anti-K_v by plasma exchange, immunosuppressive treatment or spontaneous fall [126, 128–130].

5.2. Human-Immunodeficiency-Virus-Type-1 (HIV-1-) Associated Dementia (HAD). HAD is a severe and debilitating form of HIV-1-associated neurocognitive disorders (HANDs). Over 40 million people worldwide are infected by HIV and 20–30% of them displayed symptoms of HAD. This disorder is characterized by cognitive deficits, motor disturbances, and behavioral abnormalities (for reviews see [131, 132]).

In individuals with HAD, the cognitive deficits could be result of K_v channels dysfunction [133, 134]. The brain cortex in individuals with HAD revealed overexpression of genes coding for KCNs that prolong afterhyperpolarization [135].

This participation of KCN in memory in the clinical condition caused by HIV-1 is supported by experimental results. Injection of HIV-1-infected human monocyte-derived macrophages into brain of immunodeficient mice, a model of human HIV-1-encephalitis (HIVE), impaired both long-term potentiation in hippocampus and spatial learning [136, 137]. Interestingly, these two damages are reversed when the HIVE mice received systemic administration of 4-AP, a KCN blocker aforecited [138].

5.3. Schizophrenia. Schizophrenia is a neuropsychiatric disorder that affects approximately 0.8% of the world population. It is characterized by the presence of several symptoms that can be grouped into two categories: positive and negative symptoms as a function of normal behavior. Positive symptoms (in addition to normal behavior) include hallucinations, delusions, and disorganized thoughts. Negative signs (absent in normal behavior) consist of anhedonia and social withdrawal [139, 140].

Schizophrenia is associated with cognitive deficits [141]. Weickert et al. [142] found that 51% of 117 patients with schizophrenia and decline in intelligence quotient (IQ) also exhibited deficits of executive function, memory, and attention. Deficits of executive function and memory also were found in 71% of 73 individuals with schizophrenia that showed intellectually compromised or deteriorated [143].

This memory deficit observed in patients with schizophrenia may be also consequence of dysfunction of potassium channels. Early life exposure of rodents to maternal separation or social isolation is an animal model for schizophrenia [144]. Quan et al. [145] observed deficits in learning and memory in postweaning isolation-reared rats similar to individuals with schizophrenia. When compared to the housed rats, the isolated ones performed worse in probe trials and memory retention tests in Morris water maze. Interestingly, these researchers found that the amplitudes of hippocampal voltage-dependent transient potassium A-type ($I_{(A)}$) currents were enhanced, and the steady inactivation curve of $I_{(A)}$ currents was shifted towards positive potential by CSF of isolated rats. These K^+ currents regulate action potential backpropagation and the induction of specific forms of synaptic plasticity, which is thought to underlie learning and memory [146, 147]. Therefore, Quan et al. [145] suggest that the mechanism by which early stressful experience leads to long-lasting consequences for spatial memory involves hippocampal potassium ion channel currents.

5.4. Alzheimer's Disease. Disorders that impair mental abilities are among the most feared result of aging. Among the kind of dementia, Alzheimer's disease (AD) represents the substantial majority of cases. AD is a progressive neurodegenerative disease characterized by loss of function

and death of neurons in various areas of the brain, leading to loss of mental functions such as memory and learning. The clinical diagnosis of AD is usually done when the memory loss speeds up, and other behavioral and cognitive symptoms appear, mainly because the failure in the capacity to remember ordinary facts of everyday life is easily dismissed as normal aging. The episodic memory is related to the hippocampus and the interconnections circuits between this area and cortex goes through changes during aging that are highly susceptible to neurodegeneration in AD. Unfortunately, by the time of AD diagnosis, prominent neuronal loss has already occurred in the entorhinal cortex (EC), the brain's interface between the hippocampal formation and neocortex. It is worthy to mention that neurodegeneration in EC does not occur in healthy brains undergoing aging, and as structural and functional measures of the EC, dentate gyrus and CA3 region circuit display a progressive change on the course to AD. This circuit could represent a suitable target to therapies aimed to modify the disease progression [148].

In addition to the decreased number of acetylcholine (Ach) receptors in the basal forebrain cholinergic neurons (BCFN), $K_v3.1$ and $K_v2.1$ have been implicated to AD. The use of immunohistochemical techniques showed that both KCNs are expressed in the BCFN [149]. BFCN voltage-gated KCNs regulate Ach release and may participate in BFCN neurodegeneration in the course of AD.

The accumulation of amyloid beta plaques is an AD characteristic. It seems that these plaques form calcium-conducting ion channels that cause rapid neurodegeneration due to calcium overload [150]. Toxins acting as Ca^{2+} channel blockers, such as ω-agatoxin, attenuate the increases in Ca^{2+} concentration in isolated hippocampal nerve endings in the rat [149].

$I_{(A)}$ currents and the KCN behind these currents seem to have a role in AD. KCNs responsible for $I_{(A)}$ currents have been involved in the onset of long-term potentiation in mammalian neurons, which is thought to underlie learning and memory [146, 147]. A change in the steady-state properties of the $I_{(A)}$ current was showed in the amyloid-treated *Drosophila* cholinergic neurons which was sufficient to increase the threshold for the initiation of repetitive firing [151]. In this study, specific KTxs of the $K_v4.2$ channel (phrixotoxin-2) and K_v1 channel (α-dendrotoxin) were used to determine which channels triggered these currents in the *Drosophila* model. It had been shown that treatment of cholinergic neurons with amyloid peptide altered the kinetics of the current and caused a decrease in neuronal viability [152].

Brain microglia and their KCNs are also important to AD. These cells are activated to produce a "respiratory burst," which produces reactive oxygen species (ROS) that cause the death of target cells in pathological states [153]. However, ROS generated by microglia contribute to the death of neurons in neurodegenerative conditions such as Alzheimer's and Parkinson's diseases, HIV and prion infection, and multiple sclerosis (reviewed by [154]). $K_v1.3$ and $K_{Ca}2$ and $K_{Ca}4$ are required for the respiratory burst and ROS formation in cultured microglia, and the inhibition of $K_v1.3$ by agitoxin-2 prevented neuronal killing by the microglia [155]. Moreover, charybdotoxin and α-dendrotoxin, all $K_v1.3$ blockers, reduced neuron killing by microglia as shown by transwell cell-culture system [156].

5.5. Multiple Sclerosis. Multiple sclerosis (abbreviated MS, known as *disseminated sclerosis* or *encephalomyelitis disseminata*) is an inflammatory disease. In MS, the fatty myelin sheaths around the axons of the brain and spinal cord are destroyed, leading to demyelination and scarring as well as a broad spectrum of signs and symptoms such as muscular weakness, loss of coordination and speech, visual disturbances, and cognitive disability. MS is a chronic degenerative disease and the most common neurological cause of disability in young adults in industrialized societies [157]. The major disabling aspect of MS is the cognitive impairment, which is characterized primarily by memory loss, attention deficits, slowed information processing, and failure in executive function [158].

Some MS symptoms may be closely dependent upon changes in KCN [159]. $K_v1.1$ and $K_v1.2$ are specifically found in paranodal regions of axons [160]. KCN blockers such as aminopyridines (APs) when applied *in vitro* to demyelinated axons are able to restore the conduction and also to potentiate synaptic transmission [161–164]. Because of that, much attention has been given to K_v blockers as a prospective agent for MS and other neuropathies treatments [165, 166].

Moreover, MS is a chronic inflammatory autoimmune disease and the central role of T lymphocytes, and their $K_v1.3$ and $K_{Ca}1.1$ channels, in its pathogenesis has been largely evidenced [167–169]. Kaliotoxin, which blocks the lymphocyte $K_v1.3$ and the neuronal $K_v1.1$ channels, ameliorates the symptoms of adoptive experimental autoimmune encephalomyelitis (EAE) in rats [170], a widely used model for the human MS disease [167]. It is worthy to mention that 4-AP has been used to manage some of the symptoms of MS [171, 172]. 4-AP was approved by the US Food and Drug Administration (FDA) on January 22, 2010 for the treatment of MS [173].

5.6. Parkinson's Disease. Parkinson's disease (PD) is a slowly progressive degenerative disorder of the CNS and is characterized by slowness or deficiency of movement (bradykinesia), rigidity, postural instability, and tremor primarily while at rest. The motor symptoms of PD result from the death of dopamine-generating cells in the substantia nigra, a region of the midbrain. In the early stage of the disease, the most obvious symptoms are those that are movement-related, such as shaking, rigidity, slowness of movement, and difficulty with walking and gait. With time, cognitive and behavioral problems may arise, commonly followed by dementia in the advanced phase of the disease. PD is more common in the elderly, with most cases occurring after the age of 50 (for review see [174, 175]).

Potassium channels have been implicated in the pathogenesis of PD. K_{ATP} channels comprised of $K_{ir}6.2$ and Sur1 subunit are abundantly expressed in dopaminergic neurons of substantia nigra [176]. K_{ATP} directly couple the

cellular metabolic state to membrane excitability. It has been shown that activity of the mitochondrial respiratory chain complex I (CXI, also known as NADH: ubiquinone reductase) is reduced in PD patients, strongly suggesting that metabolic stress is an important trigger factor for the PD neurodegeneration. Dopaminergic midbrain neurons express different types of K_{ATP} channels mediating their differential response to the inhibition of mitochondrial complex I [177]. The use of CXI inhibitor leads to the activation of SUR1/K_{ir}6.2-containing K_{ATP} channels, which results in a membrane hyperpolarization of the dopaminergic neuron that is associated with a complete loss of spontaneous activity [178]. Studies of dopaminergic midbrain neurons in the *weaver* mouse, a genetic mouse model of dopaminergic degeneration similar to that in PD [179], sustain the proposal of K_{ATP} channel activation as a neuroprotective strategy. Up to now, no K_{ATP} channel activators have been described from arthropod venom.

5.7. Epilepsy. The term epilepsy refers to a group of neurological disorders, clinically diverse and with multiple etiologies, characterized by paroxysmal brain discharges referred to as spontaneous recurrent seizures [180, 181]. Epilepsy is the second most common neurological disorder (after stroke), affecting 1-2% of the world's population [182]. Failure to treat epilepsy causes neurobiological, psychological, and social consequences for the patient [183], particularly cognitive impairment [184, 185]. Age of onset of epilepsy, type and duration of seizures, as well as the antiepileptic drug, therapy are strongly related to cognitive dysfunction present in the syndrome [186–188].

Clearly, ion channels are critical for regulating excitability and, contribute significantly to epilepsy pathophysiology. In a recent article, N'Gouemo [189] related that the loss-of-function large conductance K_{Ca} channels mutations contribute to neuronal hyperexcitability that can lead to temporal lobe epilepsy, tonic-clonic seizures and alcohol withdrawal seizures, and blockage of these channels can trigger seizures and status epilepticus.

Despite its availability, conventional and "new generation" antiepileptic drugs (AEDs) are commonly associated with side effects, which can vary in frequency and severity [190]. In addition, AEDs are ineffective in controlling seizures in one-third of patients (drug-resistant), reaching 70% in patients with temporal lobe epilepsy, which is the more frequent epilepsy in adults and it is characterized by degeneration of limbic structures (sclerotic hippocampus), directly involved in different memory processes and in their modulation [191–194]. It is noteworthy that cognitive deficits represent a serious neuropsychological problem in people suffering from temporal lobe epilepsy.

Several studies suggest that KCN may be an important new class of targets for anticonvulsant therapies [195, 196], especially for refractory epilepsy. New drugs that act on potassium channels have been provided and approved by the FDA and the European Union, for example, ezogabine (retigabine). *In vitro* studies indicate that ezogabine acts primarily by opening neuronal K_v7.2–7.5 channels [197].

6. Arthropod Toxins: Therapeutic and Study Tools

Given that the dysfunction of K^+ channels has significant contribution in cognitive deficits of several neurological clinical conditions, the drugs that modulate their activity became important allies in the study and treatment of these pathologies. Due to the wide presence and diversity of potassium channels in the brain, these therapeutic agents need to be specific and potent. In this way, the toxins from arthropods turn out to be excellent candidates.

6.1. Potential of KTxs as Pharmacological Tools. KTxs that act on KCN responsible for $I_{(A)}$ currents are of particular interest. These channels regulate firing frequency, spike initiation and waveform in excitable cells and may contribute to specialized functions, such as learning, memory, and behavior [146, 147]. According to the cell type and to the molecular heterogeneity, $I_{(A)}$ currents exhibit a wide diversity of physiological properties. KCNs of subtype K_v4 participate in the generation of $I_{(A)}$ currents in the cerebellum granular cells [198], neostriatal cholinergic interneurons [199], and hippocampal interneurons [147]. K_v2 channels seem to be equally important for the existence of $I_{(A)}$ currents in neocortical pyramidal neurons [200, 201]. Therefore KTxs that influence the $I_{(A)}$currents, or more specifically in K_v2 and/or K_v4 channels activity, become potential pharmacological tools for studying the participation of KCN in learning memory and its dysfunctions.

Many scorpion KTxs influence the $I_{(A)}$currents. Noxiustoxin (NTX), from the venom of the Mexican scorpion *Centruroides noxius* Hoffmann [202], and discrepin, toxin from the Venezuelan scorpion *Tityus discrepans* [203], belong to α-KTx15 subfamily. Toxins of this subfamily are reported to affect the $I_{(A)}$ currents [198]. Interesting, discrepin blocks irreversible K^+-channels of rat cerebellum neurons [203]. BmTX3 (systematic name α-KTx 15.2) is toxin isolated from venom of Manchurian scorpion *Mesobuthus martensi* is Karsch. This peptide blocked (Kd = 54 nM) completely the $I_{(A)}$ current of striatum neurons in culture, whereas the sustained K^+ current was unaffected. The labeled synthetic toxin (^{125}I-sBmTX3) was found in the striatum, hippocampus, superior colliculus, and cerebellum in the adult rat brain [204]. BmTx3B (Martentoxin, systematic name alpha-KTx 16.2) is another toxin isolated from *Mesobuthus martensi* Karsch. It was tested on two types of voltage-dependent potassium currents recorded from dissociated hippocampal neurons of neonatal rat in whole-cell voltage-clamp mode. BmTx3B selectively inhibited the delayed rectifier potassium current ($I_{(K)}$), without affecting the $I_{(A)}$ current [205]. Therefore these scorpions KTxs are candidates to become tools for the study of KCN in physiological and pathological mechanisms of learning and memory.

As mentioned above, the spider venom is a source of KTxs that act on K_v2 and K_v4 channels. The first spider toxins described as KCN blockers were the hanatoxins 1 and 2 (HaTx 1 and 2) of the theraphosidae spider *Grammostola spatulata* [206]. Despite the marked differences in their

primary sequence, both blocked the $K_v2.1$ channel with Kd of 42 nM. HaTxs block K_v1 and K_v3 channels, while the *shal*-related channel (type K_v4) is sensitive to the toxin. The heteropodatoxins (HpTx 1–3), isolated from the venom of *Heteropoda venatoria* (Sparassidae), blocked the conductance of $K_v4.2$ but not of $K_v1.4$, in a voltage-dependent manner. Other toxins that act on KCN were isolated from the venom of the Theraphosidae *Phrixotrichus auratus*. The phrixotoxins (Patx 1 and 2) specifically block $K_v4.3$ and $K_v4.2$ currents at nanomolar concentrations altering the gating properties of these channels by interacting with the voltage sensor. The subfamilies of *shaker* (K_v1), *shab* (K_v2), and *shaw* (K_v3) were not inhibited by PaTxs [207]. The stromatoxin1, ScTx1, isolated from tarantula *Stromatopelma calceata*, was the first high-affinity inhibitor described for the $K_v2.2$ channel. ScTx1 also inhibited $K_v2.1$ channels, $K_v4.2$ and $K_v2.1/K_v9.3$ heterodimer [208]. HmTx 1 and 2, peptides purified from the tarantula spider *Heteroscodra maculata*, inhibited potassium currents associated with subtypes K_v2 [206]. Guangitoxin-1E (GxTX-1E) is a potent gating modifier peptide of K_v2 channels isolated from the venom of the tarantula *Plesiophrictus guangxiensis* [209]. Other spider toxins targeting the K^+-channel voltage sensor include heteroscodratoxins (HmTx1,2), which target K_v2 and K_v4 channels [206]; TLTx1–3, which preferentially inhibit K_v4 channels [210]; PhTx3-1, which inhibits the outward rectifier A-type K^+-channel [211]. SGTx1 (Kappa-theraphotoxin-Scg1a) is a peptide toxin isolated from the venom of the aggressive African Theraphosidae *Scodra griseipes* that has been shown to inhibit outward K^+ currents in rat cerebellar granule neurons [64]. Functionally, SGTx1 reversibly inhibits potassium currents in oocytes expressing $K_v2.1$ channels and acts by shifting the activation of the channel to more depolarized voltages [212]. Finally, phrixotoxin-1, a peptide purified from the venom of the tarantula *Phrixotrichus auratus,* is a specific and potent blocker of $K_v4.3$ [207]. So there are many spider KTxs that are potential tools for the study of KCN in learning and memory and its dysfunction.

Toxins that act on large-conductance Ca^{2+}-activated K^+-channels ($K_{Ca}1.1$ or BK) and small-conductance Ca^{2+}-activated K^+ channel ($K_{Ca}2.1$ or SK1, $K_{Ca}2.2$ or SK2, and $K_{Ca}2.3$ or SK3) are important too. Neuronal firing is also regulated by $K_{Ca}1.1$ and $K_{Ca}2$ which constitute an exclusive family of ion channels which combine intracellular chemical changes and electric signaling [213, 214]. $K_{Ca}1.1$ are homologous to K_v channel α-subunits, but possess additional hydrophobic segments forming an extracellular N-terminal and a long intracellular C-terminal that holds one of the Ca^{2+}-binding sites [215]. It has been reported that K_{Ca} channels are involved in regulation of neocortex pyramidal cell excitability [216, 217]. Therefore KTxs that influence the K_{Ca} channels become also potential pharmacological tools.

K_{Ca} channels are blocked by many scorpion toxins. Martentoxin, purified from *Mesobuthus martensi* Karsch venom, is able to block $K_{Ca}1.1$ currents in rat hippocampal neurons [218]. This KCN is also inhibited by slotoxin from *Centruroides noxius* [219] and noxiustoxin from *Centruroides noxius* [220]. Slotoxin is described not only as a potent and selective blocker, but it also can differentially inhibit $K_{Ca}1.1$ channels, depending on the presence of β-subunits [219] and on the α-splice variant [221]. $K_{Ca}2$ channels are also blocked by different scorpion toxins. For example, there are scyllatoxin (leiurotoxin I) isolated from *Leiurus quinquestriatus hebraeus* [222] and tamapin from *Mesobuthus tumulus* [223]. This last toxin blocks $K_{Ca}2$ channels in pyramidal neurons of the hippocampus as well as in cell lines expressing distinct $K_{Ca}2$ channel subunits, displaying a remarkable selectivity for $K_{Ca}2.2$ (IC50 = 24pM) versus $K_{Ca}2.1$ ($\cong$1750-fold) and $K_{Ca}2.3$ ($\cong$70-fold) channels [223]. These data reinforce the potential of scorpion KTxs as pharmacological tools.

As has been evident, multiple KCNs participate in the regulation of cellular events behind the phenomena of learning and memory and their dysfunctions. Therefore, it is necessary a varied arsenal of pharmacological tools to investigate the role of each potassium channel in these cognitive processes. It also became clear that scorpions and spiders KTxs can provide this diversity of tools.

7. Conclusion

The KCNs are components of the mechanisms responsible for learning and memory. Its diversity and wide distribution in the brain make the action of KCN during the formation of learning and memory retrieval to be complex and variable. To investigate the role of each subtype of KCN in these phenomena, we need also a wide variety of pharmacological tools, specific and potent for KCN. The toxins from the venoms of arthropods can be such tools. These precise potassium channel toxins may not only have an important contribution to uncover the processes underlying learning and memory, but can also become therapeutic agents for many diseases and disorders of the CNS.

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Review Article

Potassium Channels Blockers from the Venom of *Androctonus mauretanicus mauretanicus*

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K^+ channels selectively transport K^+ ions across cell membranes and play a key role in regulating the physiology of excitable and nonexcitable cells. Their activation allows the cell to repolarize after action potential firing and reduces excitability, whereas channel inhibition increases excitability. In eukaryotes, the pharmacology and pore topology of several structural classes of K^+ channels have been well characterized in the past two decades. This information has come about through the extensive use of scorpion toxins. We have participated in the isolation and in the characterization of several structurally distinct families of scorpion toxin peptides exhibiting different K^+ channel blocking functions. In particular, the venom from the Moroccan scorpion *Androctonus mauretanicus mauretanicus* provided several high-affinity blockers selective for diverse K^+ channels (SK_{Ca}, $K_v4.x$, and $K_v1.x$ K^+ channel families). In this paper, we summarize our work on these toxin/channel interactions.

1. The Scorpion Venom Content

Scorpion venoms are very complex mixtures of molecules, constituting a diverse, naturally occurring peptide library, with most peptides displaying different kinds of biological activity [1, 2]. These peptides can specifically bind to a variety of pharmacological targets, in particular ion channels, resulting in neurotoxic effects. Toxins modulating Na^+, K^+, Ca^{++}, and Cl^- currents have been described in scorpion venoms [2].

Toxins that are highly lethal for mammals modify voltage-gated Na^+ (Na_v) currents in excitable cells and are referred to as "Na_v channel long-chain toxin." These toxins are single-chain, small, basic peptides (60- to 75-amino-acid residue chain generally folded by four disulfide bridges). They have been described as α- or β-toxins due to their binding site on Na_v channels as well as to their pharmacological effects [1, 3]. α-Toxins bind in a voltage-dependent manner on the voltage sensor of the Na_v channel domain IV and inhibit the inactivation phase of the action potential. β-Toxins act on the channel activation phase by binding to extracellular loops located preferentially on the voltage sensor of the Na_v channel domain II (but also occasionally of domain III) [1, 3–6].

Another class of scorpion toxins has also been widely studied, even if these toxins are devoid of serious lethal effect. They block different K^+ channel subtypes (some of them in the picomolar range) and are so-called "K^+ channel toxins" [1, 2, 7]. They are usually shorter than Na_v channel toxins but are structurally closely related to them. These Na_v and K^+ channels toxins share a common dense scaffold typically formed by an α-helix and a β-sheet stabilized by disulfide bridges [8].

Several components of scorpion venom that act on Cl^- and Ca^{++} channels have also been described [2]. However, they have little or no influence on the venom toxicity for mammals.

This overview will focus, in particular, on our work done on the K^+ channel blockers purified from the *Androctonus mauretanicus mauretanicus* venom, which were among the first K^+ channel blockers characterized from scorpion venoms.

2. K^+ Channel Blockers from Scorpion Venoms

K^+ channels constitute a ubiquitous family of transmembrane proteins which play a key role in the regulation of a wide variety of physiological processes involved in cell excitability, including regulation of heart beat, muscle contraction, neurotransmitter release, hormonal secretion, signal transduction, and cell proliferation [9]. Multiple combinations of K^+ channels result from the ability of their subunits to coassemble as tetramers, thus considerably increasing the total number of functionally distinct K^+ channels. According to their functional and gating properties, K^+ channels have been first divided into four groups: voltage-activated, Ca^{2+}-activated, inward rectifier, and two-pore K^+ channels [10]. Their 3D architecture has now been depicted by X-ray crystallography [11].

K^+ channel blocker's toxins (KTxs) from scorpion venoms are short peptides, which are made usually of about 28–40-amino-acid residues reticulated by three or four disulfide bridges, forming compact and resistant molecules [7]. They have been invaluable tools for understanding the physiological role of K^+ channels and have been exploited to gain insights into the structure of the channel pore that they occlude via electrostatic and hydrophobic interactions [12, 13]. They block K^+ channels from the extracellular side and bind to their outer vestibules. In most cases, they possess at least two functionally crucial residues: examples include a lysine residue that plugs the channel pore with its side chain and a hydrophobic residue that strengthens the interaction between the toxin and its target. These residues are found in very low concentrations in the venoms (from 0.01 to 1% by weight of crude venom) and have almost no toxic effects in mice when injected by subcutaneous route. However, some of them could be very toxic following direct intracerebroventricular injection.

Based on primary amino acid sequences and cysteine pairing, KTxs have been classified into four families, the α-, β-, γ-, and κ-KTx [2, 7]. So far more than 120 KTxs, ranging from 23 to 64 amino acids, have been isolated and sequenced. Most of their structures exhibit a common minimal motif, named the "Cystein-Stabilized-Helix" (CSH). As found in Buthidae scorpions, long and short toxins consist of one α-helix and two or three β-strands, in which two disulfide bridges covalently link a segment of the α-helix with one strand of the β-sheet structure [8]. Even if their amino acid sequences are different, the conserved peptide fold allows insertions, deletions, and mutations conferring to the toxins diverse selectivity and affinity for their target. Only the recently characterized κ-KTxs are formed by two parallel α-helices linked by two disulfide bridges [14].

The α-KTx family is the largest one and is divided into at least 22 subfamilies, defined according to the primary sequence alignments of the toxins. Each member has diverse, specific blocking activities against voltage-gated (K_v) and calcium-activated (K_{Ca}) channels. Most of the α-KTxs bind to the ion channel vestibule through the β-sheet side of their structure.

Other, longer peptides, with 45- to 68-amino acid residues and cross-linked by three disulfide bridges, have been characterized and classified as the β-KTx family. They possess two structural and functional domains: an N-terminal α-helix (with cytolytic or antimicrobial activity like the insect defensins) and a tightly folded C-terminal region with the CSH motif (displaying K^+ channel-blocking activities [15]). Finally, the γ-KTx family was described as specifically targeting hERG channels [16].

3. The *Androctonus mauretanicus mauretanicus* Venom

Scorpion stings in Morocco are the primary cause of envenomation and constitute a largely underestimated health problem. An epidemiologic study of four regions of the Moroccan Kingdom, where scorpion stings are prevalent, showed that the stings are mainly due to the black scorpion *Androctonus mauretanicus mauretanicus* (83% of the reported cases). Children, in desert areas far from medical centers, were the primary victims, with casualty rates up to 8% in those under ten years old. The *Androctonus mauretanicus mauretanicus* venom is one of the most toxic Buthidae venoms ever described (its median lethal dose ranges from 0.05 to 0.2 mg/kg by subcutaneous injection in mice) and immunotherapy remains the treatment of choice [17].

Previous fractionation studies of the venom allowed identification of several toxins that are active on different Na_v or K_v channels [18–22]. At least several major proteins, considered highly toxic to mice, have already been purified and chemically and pharmacologically characterized as classical α-toxins [18, 19]. All together, these toxins represent about 28% of the absorbance (wavelength 280 nm) of the crude venom and 73% of the total lethality for mice. The most represented and the most lethal classical α-toxin Amm V alone constitutes 11% of the absorbance and 47% of the total lethality.

However, other toxins isolated from the *Androctonus mauretanicus mauretanicus* venom gained popularity as powerful tools because they have displayed some of the highest binding affinity and specificity for K^+ channels. They have been extensively used to investigate the mechanisms of ion conduction and channel selectivity, as well as the architecture of the pore region. Finally, significant advances have been made by using solid-state NMR data to construct 3D structures from Kaliotoxin (KTX) in complex with a chimeric K^+ channel KcsA-K_v1.3 [13]. These studies allowed direct investigation of the molecular rearrangements associated with KTX binding on both the channel selectivity filter and the KTX itself.

A recent MALDI-TOF mass spectrometry (MS) study has provided new information about the molecular composition of scorpion venom [23]. Through the developments in proteomics, MS is now widely used for accurate and sensitive determinations of molecular masses and identification of heterogenous complex mixtures such as crude venoms. Using an offline MALDI-TOF/MS analysis, we were able to determine the molecular masses of about 70 to 80 different compounds ranging between 3000 and 8000 Da in an *Androctonus mauretanicus mauretanicus* venom obtained

under manual stimulation from a pool of animals kept alive at the "Pasteur Institute" of Casablanca, Morocco. Figure 1 presents the snapshot of this crude venom.

4. The Smallest Toxins Identified so Far in the *Androctonus mauretanicus mauretanicus* Venom: P01 (α-KTx8) and P05 (α-KTx5), Ligands of SK_{Ca} Channels

P01 (α-KTx 8.1 subfamily, 3177 Da) is devoid of significant toxicity in mouse [26]. This toxin of 28-amino-acid residues, which is the smallest K^+ channels ligand in the *Androctonus mauretanicus mauretanicus* venom, is slightly negatively charged with acidic amino acids localized at the beginning of the β-turn and extending along the α-helix (Figure 2(a)). Surprisingly, P01 presents exactly the same sequence in *Androctonus mauretanicus mauretanicus, Androctonus australis*, *Androctonus amoreuxi*, and *Buthus occitanus* venoms. Usually, the polymorphism among scorpion toxins is so high that their sequences vary from a scorpion subspecies to another, even in the same structural, pharmacological, and immunological family. P01 is a poor-affinity ligand (300 nM) for the apamin-binding site on rat brain synaptosomes. In comparison, apamin purified from the bee venom displays a very high affinity ($K_d = 8$ pM) for its target, that is, the small-conductance calcium-activated K^+ channel (SK_{Ca}) channel so-called apamin-sensitive channel. Since the first P01 characterization, other closely related analogs have been purified from other Buthidae venoms. Among them, one is particularly interesting because it shares 89% of identity with P01. This analog, called OdK1, was purified from the Iranian scorpion *Odontobuthus doriae* (α-KTx 8.5 subfamily). OdK1 is able to block $K_v1.2$ channels expressed in oocytes with a median inhibition concentration (IC_{50}) value of 183 nM and has no effect on $K_v1.1$, $K_v1.3$, $K_v1.4$, $K_v1.5$, and the *Shaker* channels [27]. It is important to mention that P01 has not been tested yet on $K_v1.2$ channels in electrophysiological experiments. P01 was only shown unable to compete with ^{125}I-KTX (a $K_v1.1$ and $K_v1.3$ and not $K_v1.2$ blocker) bound to its receptor on rat brain synaptosomes. Thus, we cannot totally exclude that P01 could exhibit a poor $K_v1.2$ blocking activity. In the same line, OdK1 activity on SK_{Ca} channels has not been tested yet either.

In contrast to P01, P05, another toxin from *Androctonus mauretanicus mauretanicus,* is a high-affinity selective ligand ($K_d = 100$ pM) for the apamin-binding site on rat brain synaptosomes, and it has no effects on BK_{Ca} or K_v channels. P05 is a 31-amino-acid long peptide (3415 Da), which belongs to the α-KTx5.x's family (as Leiurotoxin1 or Scyllatoxin from the venom of the scorpion *Leiurus quinquestriatus Hebraeus*) (Figure 2(b)) [29, 30]. The toxins from this family have only two-stranded β-sheet because of their short N-terminal side [28]. P05 is highly toxic (14 ng, i.e., 4 pmols for a 20 g mouse) and leads to an epileptic behavior when injected in mouse by the intracerebroventricular route. P05 possesses a short stretch of four amino acids similar to the one in the apamin sequence, which constitutes a highly positively charged region in the

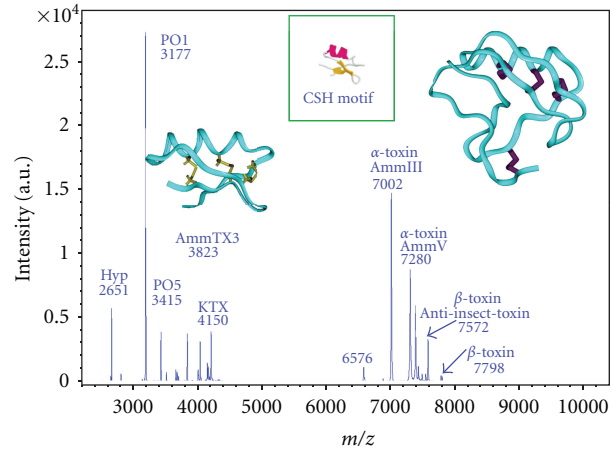

Figure 1: MALDI-TOF mass spectra of pool venom manually extracted from *Androctonus mauretanicus mauretanicus*. K^+ channel blockers and voltage-gated Na^+ channels modulators are indicated. P01 and P05, blockers of SK_{Ca} channels; AmmTX3, blockers of K_v4 and hERG channels; KTX, $K_v1.1$, and $K_v1.3$ blockers. Amm III and Amm V are major lethal α-toxins; β-toxins specific for insects are also mentioned. The 3D structure of KTX (left) and of the α-toxin of reference AaH II from *Androctonus australis* (right) are shown [13, 24, 25]. The Cys-Stabilized-Helix (CSH) motif is shown in inset [8].

α-helix (containing in particular two Arg residues, Arg_7 and Arg_9) exposed to solvent. Extensive structure-function studies using chemically synthesized analogs revealed that these two Arg were critical for P05's interaction with its target as described for apamin [31]. When Arg were replaced by Lys, the activity dropped by a factor of one hundred, and when they were replaced by Ile, the affinity decreased even more to a Kd value in the micromolar range. Moreover, α-amidation of His in P05 C-terminal conferred a large gain of function leading to an almost irreversible binding to its receptor [21]. When the apamin C-terminal residue is in the free carboxylic form, only 0.06% of the pharmacological activity of the native C-amidated apamin is retained [32]. Both Arg and C-terminal His create a strong positive electrostatic potential, which drives the toxin to negative residues (Asp) of the channel pore. Using homology-modeling models of SK channels (rsk1, rsk2, and rsk3) and Brownian dynamics methods, the recognition between P05 and its targets was investigated [33]. It was found that the rsk2 channel, presenting the highest frequencies and lowest electrostatic interaction energies, was the most favorable target for P05 binding, while rsk3 was intermediate, and rsk1 was the least favorable. From the P05-rsk2 complex model, it was shown that P05 probably locates around the extracellular pore of SK channels and assures the contact with rsk2 channel using critical basic amino acid residues in its α-helix: Arg_6 (P05)-Asp_{364} (SK), Arg_7 (P05)-Asn_{368} (SK), and Arg_{13} (P05)-Asp_{341} and Asp_{364} (SK). Further refinements of P05-rsk2 complex model using molecular mechanics showed that six hydrogen bonding interactions exist between P05 and the rsk2 channel. These simulation results were in good agreement with our previous *in vivo* binding experiments and could explain

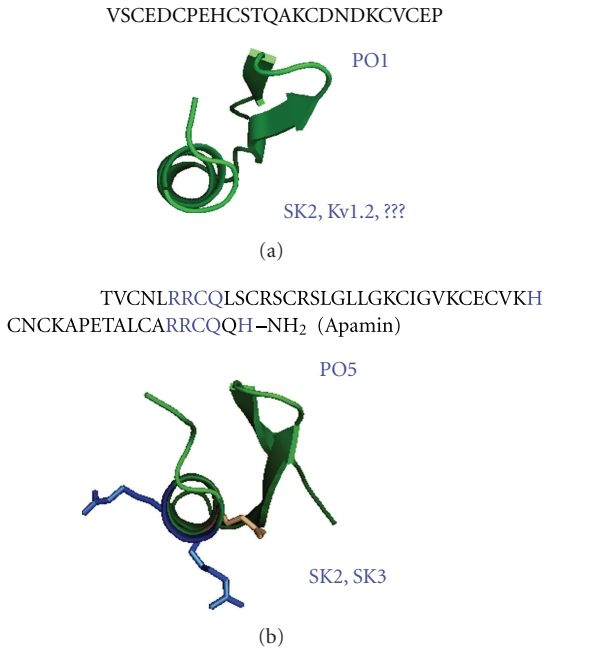

Figure 2: Amino acid sequences and 3D structures of P01 and P05. (a) Amino acid sequence (single letter notation) and 3D structure of P01 [26, 28]. (b) Amino acid sequence (single letter notation) of P05 compared to that of the bee venom apamin (18 mers peptide). Amino acids common to P05 and apamin and crucial for the SK_{Ca} blockade are in blue. These amino acids are materialized on the 3D structure of P05, view by the α-helix axe. The two positive Arg are in blue and the neutral Gln is in brown.

the interaction between P05 and SK channels at the level of the molecular structure.

5. The Most Noted K^+ Channel Blocker Characterized in the *Androctonus mauretanicus mauretanicus* Venom: Kaliotoxin (α-KTx3 Subfamily)

The Kaliotoxin (KTX, 4150 Da) purified from the venom of the *Androctonus mauretanicus mauretanicus* was the first identified member of the α-KTx3.1 subfamily, which is currently composed of 13 highly homolog members sharing more than 75% sequence identity. KTX was first described as a BK_{Ca} low-affinity blocker [20], but further analysis showed that KTX blocked more specifically $K_v1.3$ channels with a very high affinity ($K_d = 10$ pM) and $K_v1.1$ with a much weaker affinity (in the 10 nM range). KTX also exhibited a functional blockade of Ca^{2+}-activated K^+ Gardos channel from rabbit or human red blood cells with a median inhibition concentration value of 5 nM. Interaction between this channel and other peptide toxins indicated that the Gárdos channel was pharmacologically different in several interesting ways from the other Ca^{2+}-activated K^+ channels [34]. Interestingly, KTX was not toxic in mice by subcutaneous injection up to 500 micrograms (100 nmols), but intracerebroventricularly injected mice showed tremor, paralysis, and death with a median lethal dose of only 24 ng of KTX (about 6 pmol for a 20 g mouse).

KTX was then widely used by different international groups to probe the vestibule topology from the lymphocyte $K_v1.3$ channel. The homotetrameric $K_v1.3$ channel controls the resting potential membrane in T cells and plays a crucial role in human T-lymphocyte activation [35]. Its inhibition causes depolarization and an attenuation of the rise in the intracellular Ca^{2+} concentration that is required for T-cell activation. Accordingly, $K_v1.3$ channel is a good therapeutic target for the development of immunosuppressant drugs. It is important to note that $K_v1.3$ channel is a key mediator of multiple sclerosis, type 1 diabetes mellitus, and rheumatoid arthritis pathologies [36]. In experimental autoimmune encephalomyelitis (EAE) rat, animal model for multiple sclerosis, KTX was capable of immunosuppressant activity *in vivo*. Addition of KTX during Antigen/T-cell activation led to a large reduction of the T-cell proliferative response, a decreased encephalitogenicity of T cells, and finally improved the symptoms of EAE [37].

A new therapeutic approach for inflammatory bone resorption by targeting $K_v1.3$ was also investigated and the potential effect of KTX was tested on inflammatory lesions of periodontal disease. These lesions contain abundant activated/memory T and B cells, which control immunological interactive networks and accelerate bone resorption. Systemic KTX administration finally resulted in an 84% decrease of the bone resorption. These results suggested that KTX could also constitute a potential therapy to prevent alveolar bone loss in periodontal disease [38].

Monoiodinated derivative of KTX (^{125}I-KTX) binds specifically to rat brain total membranes with a maximal binding capacity of about 14 fmol/mg of protein and with a high affinity ($K_d = 80$ pM). The distribution of ^{125}I-KTX binding sites in rat brain was first studied using quantitative autoradiography on adult brain tissue sections. A comparison with the distribution of $K_v1.1$ and $K_v1.3$ α-subunits by immunohistochemistry or *in situ* hybridization suggested that KTX recognizes channels containing these subunits [39]. Further, we used KTX injected by intracerebroventricular route to rat brain in order to investigate the involvement of K_v channels containing $K_v1.1$ and $K_v1.3$ α-subunits in olfactory associative learning and memory. KTX facilitated cognitive processes as learning, in particular in a reference representation [40]. Therefore, it is likely that KTX-sensitive K_v channels contribute to the repolarization of action potentials at presynaptic terminals of hippocampal inhibitory neurons and induce facilitation of the transmission.

Concerning the KTX structure-function relationship studies, they were first performed using synthetic analogs such as $KTX_{(1-37)}$, $KTX_{(1-37)\text{-amide}}$ and short peptides including $KTX_{(27-37)}$, $KTX_{(25-32)}$, and $KTX_{(1-11)}$. Concerning the short peptides, which corresponded to secondary structural elements, only $KTX_{(27-37)}$ and $KTX_{(25-32)}$were able to compete with ^{125}I-KTX for its receptor on rat brain synaptosomes and act as antagonists of KTX. These results demonstrated for the first time that the C-terminal region, particularly the toxin β-sheet, was involved in the interaction with the

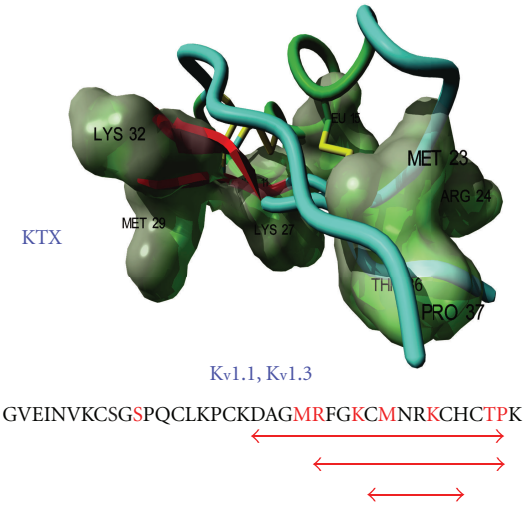

FIGURE 3: Amino acid sequence and 3D structure of KTX. The crucial amino acid residues involved in the receptor recognition are in red and are materialized on the 3D structure according to Lange et al., 2006 [13]. The α-helix is in green, the β-sheet in red. Arrows in red indicate the three peptides able to compete (K_d = 100 nM) with the ^{125}I-KTX bound to its binding site on rat brain synaptosomes [41].

receptor and the channel blockade [41]. Figure 3 summarizes these results.

Several international groups also produced KTX and numerous mutants using chemical synthesis or heterologous expression, in order to identify KTX residues implicated in the specific interaction with K_v1.3 channels. It was found that the side chain of Lys_{27} of the toxin enters deeply into the channel pore and interacts with the Asp_{402} residue of each subunit [24]. Briefly, the KTX residues involved in the interaction with the channel were Arg_{24}, Phe_{25}, Lys_{27}, Met_{39}, Asn_{30}, Arg_{31}. All these residues were present in the short peptides that we first used to demonstrate the β-sheet importance in the channel blockade. A model also predicted that Ser_{11} of the KTX made steric contacts with His_{404} and Pro_{405} of the α-subunit IV of the K_v1.3 and Thr_{36} with His_{404} of the opposing K_v1.3 α-subunit [42].

KTX binding to a chimeric K^+ channel (KcsA-K_v1.3) in proteoliposomes was investigated using solid-state nuclear magnetic resonance (ssNMR) [13]. Upon complex formation, significant chemical shift changes of the residues implicated in the specific interaction were observed for both KTX and chimeric channel. For KTX, the conformational changes involved mainly β-sheet contacts between the first and third β-strand and for the chimeric channel it affected the conformation of both the pore helix and the selectivity filter. The backbone conformation of Kcsa-K_v1.3 selectivity filter adopted a novel structure with features of both the conducting and collapsed conformation of the KcsA. The ssNMR data directly showed that Asp_{64} in the KcsA-K_v1.3 vestibule represented an important interaction site for KTX. Large chemical shift changes were seen for Gly_{77}, Tyr_{78}, and Gly_{79} in the selectivity filter and also for the side chains of Glu_{71} and Asp_{80} that form carboxyl-carboxylate pairs on the backside of the filter [13]. Finally, an enhanced backbone mobility was detected for two glycine residues within the selectivity filter that are highly conserved amongst potassium channels and are of core relevance to the filter structure and ion selectivity [43, 44].

Combination of additional ssNMR studies, dynamic simulations, and electrophysiological measurements finally revealed the complete mechanism of KTX binding to its receptor site and showed a structural link between inactivation and block of the channel. A mechanism of cooperative toxin-induced conformational changes that are structurally and functionally related to recovery from C-type inactivation, would be the consequence of the very tight interaction between KTX and Kcsa-K_v1.3. The KTX affinity was lowered by about 20-fold when the K_v1.3 channels entered in C-type inactivation probably due to changes of the interaction surface between the toxin and the channel [43–45].

All these recent ssNMR data obtained between the chimeric KcsA-K_v1.3 channel and KTX have largely contributed to decrypt the intimate interaction between a K^+ channel blocker and its target, as well as to better understand the blockade of the K^+ conduction.

6. The α-KTx$_{15}$ Subfamily: Janus K_v4.x and hERG Blockers

The first member of the α-KTx_{15} subfamily characterized was Aa1 from the *Androctonus australis* venom (3869 Da) [46]. At the primary sequence level, the toxin has an unusual N-terminal pyroglutamic acid, like Charybdotoxin and Iberiotoxin, but the rest of its sequence was totally original. Aa1 completely blocked a fast I_A-type K^+ current from cerebellum granular cells. Using whole-cell patch clamp recording on striatal neurons in culture, we selected another novel toxin, BmTX3 from *Buthus martensii*, which was also able to block a fast I_A-type K^+ current. The sustained current was unaffected with a micromolar dose of toxin whereas the I_A-type K^+ current completely disappeared independently of the membrane potential. Autoradiograms of adult rat brain sections demonstrated a highly heterogeneous distribution of ^{125}I-BmTX3 binding sites throughout the adult rat brain. High density of receptors was found in the striatum, the CA1 and CA3 field of the hippocampus, the superior colliculus, and in the granular layer of the cerebellum [47]. We then purified AmmTX3 (3827 Da), an analog of Aa1 and BmTX3, from the venom of *Androctonus mauretanicus mauretanicus* [22]. Latter, several cDNAs encoding two Aa1 isoforms, AaTX1 (3867 Da) and AaTX2 (3853 Da), were identified by PCR amplification from a venom gland cDNA library of *Androctonus australis* [48]. Also, another oligonucleotide sequence, AamTX (3751 Da), was amplified from a venom gland cDNA library of *Androctonus amoreuxi* [49]. Altogether these toxins constitute the first members of the α-KTx_{15} subfamily (Figure 4). From a pharmacological point of view, theses toxins were unable to compete with any other already described toxins purified from animal venom. However, they were toxic in mouse at high doses by intracerebral injections and induced epileptic status, which

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Aa1       ZNETNKKCQGGS-CASVCRRVIGVAAGKCINGRCVCYP
BmTX3     ZVETNVKCQGGS-CASVCRKAIGVAAGKCINGRCVCYP
AmmTX3    ZIETNKKCQGGS-CASVCRKVIGVAAGKCINGRCVCYP
AamTX*    ZVQTNKKCKGGS-CASVCAKVIGVAAGKCINGRCVCYP
AaTX1*    ZIETNKKCQGGS-CASVCRRVIGVAAGKCINGRCVCYP
AaTX2*    ZVETNKKCQGGS-CASVCRRVIVVAAGKCINGRCVCYP
Discr.    ZIDTNVKCSGSSKCVKICIDRYNTRGAKCINGRCTCYP
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Figure 4: Amino acid sequences of the toxins from the α-KTx$_{15}$ subfamily. Aa1 [46], AaTX1, and AaTX2 [48] are from *Androctonus australis*; BmTX3 [47] is from *Buthus martensii*; AmmTX3 [22] is from *Androctonus mauretanicus*; AamTX [49] is from *Androctonus amoreuxi*. Discrepin [54] is from the Venezuelan scorpion *Tityus discrepans*. Single letter notation is used for amino acid sequences. Z is pyroglutamic acid. *Indicates putative amino acid sequence deduced from cloned cDNA oligonucleotide sequence. The β-sheet consensus sequence is in grey.

could last 24 to 48 hours. Also, they all shared the same target on rat brain synaptosomes because they competed with each other for the same binding site. The nature of the K^+ channels blocked by AmmTX3 was assessed by performing whole-cell patch recording of the K^+ currents of striatal neurons and of cerebellum granular cells in culture. In all cases, AmmTX3 inactivated the transient A-current without affecting the sustained K^+ current, as observed for Aa1 and BmTX3. As well described, A-type K^+-currents result mainly from the expression of voltage-dependent K_v α-subunits (K_v1.4, 3.4, 4.1, 4.2, 4.3) or from the association of K_v β-subunit with K_v1 α-subunits [50, 51]. In the cerebellum granular cells, the voltage-gated K^+ channels K_v4 of the Shal subfamily elicits A-type currents. These channels are fast transient K^+ channels that regulate the kinetics of the action potential [51, 52]. The localizations of K_v4 subunits and their auxiliary subunits were similar to the distribution of BmTX3 binding sites in numerous regions of the brain. For example, the high density of BmTX3 binding sites in the CA1 field and CA3 field followed K_v4.2 and K_v4.3 immunostaining patterns. In the molecular layer of the dendate gyrus, the BmTX3 binding pattern was also similar to the immunoreactivity pattern of K_v4 and KChIP subunits, with a higher density in distal dendrites of the granular layer than in the more proximal dendrite areas [53].

Finally, electrophysiological analysis of mammalian cells expressing different A-type channels showed that BmTX3 completely inhibited the rapidly activating and inactivating K_v4.1 current in a voltage-independent manner. This inhibition was less effective on K_v4.2 and K_v4.3 channels and the toxin did not show any effects on other transient currents elicited by K_v1.4 and K_v3.4 [53]. Recent electrophysiological studies using acute coronal midbrain slices and AmmTX3 confirmed that the targets of the α-KTx$_{15}$ subfamily are K_v4 channels [55, Rudy B, personal communication]. Interestingly, the radioiodinated α-KTx$_{15}$ toxins bound to their receptor on rat brain neurons were not displaced by any spider toxin, which block K_v4 channels. This result suggests that these scorpion and spider K_v4 blockers have different binding sites. The α-KTx$_{15}$ toxins most probably block the K_v4 channel pore because tarantula toxins inhibit K_v4 channels by binding to the voltage-sensor paddles (crucial helix-turn-helix motifs within the voltage-sensing domains composed of the S3b and S4 helices) [56]. Moreover, deletions of the last two BmTx3 C-terminal residues (α-KTx$_{15}$ subfamily) were made to assess the role of the penultimate Tyr residue in the receptor recognition. This Tyr resembled to the canonical dyad previously proposed as necessary to block the K^+ channel conduction with high efficacy. Our results showed that the truncated toxin bound to its receptor less efficiently than the wild-type toxin (by a factor of about 10^5) and had no more channel blocking activity [57]. All these results suggest that the K_v4 channels blocked by the α-KTx$_{15}$ subfamily might have a canonical K^+ channel pore structure.

Moreover, we also described a significant hERG-blocking activity from the α-KTx$_{15}$ toxins, as previously shown for the γ-KTx peptides. AmmTx3 induced a hERG channel block with no alteration of the gating kinetics. According to a model of the γ-KTx toxin BeKm-1 from *Buthus eupeus* docked on hERG channels, the toxin BeKm-1 is above the pore entrance and none of its side chains penetrate deeply into the pore. While α-KTx members usually interact with channels through their β-sheets, γ-KTxs modulate hERG in a different way than the one proposed for the interaction between Charybdotoxin and the Shaker channel or between KTX and the K_v1.3, in which the critical Lys$_{27}$ protrudes into the pore of the channel. BeKm-1 uses its α-helix and the following turn (possessing two basic residues Lys$_{18}$ and Arg$_{20}$), to interact with hERG channel [58, 59]. In contrast to the other α-KTx$_{15}$ members, a new toxin of this subfamily isolated from the Venezuelan scorpion *Tityus discrepans,* called Discrepin (α-KTx$_{15-6}$), was also able to block an A-type K^+ current in cerebellum granular cells in culture, but was ineffective to block the hERG channel. Its amino acid sequence displays only 50% identity with the other members purified mainly from Old-World scorpion venoms (Figure 4). Discrepin C-terminal β-sheet, supposed to interfer with the K_v4 channel pore, presented a consensus amino acid sequence similar to those found in the other members (in grey on Figure 4), but its "hot spot" for hERG channel blockade was altered. After several point mutations in AmmTX3 and introduction of positive charged residues in Discrepin, it was finally demonstrated that a common "hot spot" composed of two basic residues (Arg$_{18}$ and Lys$_{19}$ near the end of the α-helix) conferred hERG blockade activity of α-KTx$_{15}$ peptides [54]. From a structural point of view, we proposed that two separate functional surfaces (A and B) could coexist on the α-KTx$_{15}$ toxins, and were responsible for two different K^+-current-blocking functions [60].

To extend our theory to other members from α-KTx subfamily, the effects of a "hot spot-" bearing toxin were tested on hERG channels and compared to the results obtained with a toxin without the "hot spot." From these studies, it was concluded that only the α-KTxs possessing the "hot spot" were able to interact with the pore of hERG channels. This pharmacophore could either be -CKKX- or -CKXK- or -CXKKX-, with C being the third Cys, X being any nonpositively charged amino acid, and K being Lys or Arg [54].

7. Conclusion

Scorpion venom still remains a proven resource for the discovery of novel biologically active compounds, especially for the pharmacologists involved in research on ionic channels. A total of about 210 α-KTx oligonucleotide or amino acid sequences are now referenced in the UniProtKB data bank, but only some of these α-KTx peptides have really been shown able to block K^+ currents. Unfortunately, very often, there is no direct evidence described for the function of the reported peptides. Some of them represent new analogs of well-known families with described channel selectivity, but others exhibit novel structural features or activities. Majority of the α-KTx effects were determined on the $K_v1.x$ subfamily or in a less extent on the Ca^{2+}-activated K^+ channels (SK_{Ca} sensitive to the bee venom apamin or BK_{Ca}). During the last two decades, we obtained a lot of results that provided new insight into the targets and the mode of action of some α-KTxs isolated from different potent North African *Androctonus* venoms. In particular, numerous studies on the *Androctonus mauretanicus mauretanicus* venom "manually" extracted, which can be considered as the physiological venom secretion, have greatly contributed to the chemical, immunological, structural, and pharmacological characterization of some highly specific K^+ channel blockers. With AmmTX3, we have enlarged the α-KTx_{15} subfamily and defined more properly its target, the K_v4 channels. KTX, from the α-KTx_3 subfamily, was finally proven to be a powerful tool used by several international teams to depict the molecular mechanisms of interaction between K^+ channels and peptide inhibitors, as well as to demonstrate that the binding of K^+ channel specific scorpion toxins does not take place only on the outer vestibule of the channel pore but also deeper into the selectivity filter. The binding involves a combination of hydrophobic, hydrogen bonding and electrostatic interactions, which induces significant structural rearrangements in both interacting molecules. It was then proposed that structural flexibility of the K^+ channel and the toxin represent an important determinant of the high specificity of toxin/K^+ channel interactions [13].

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Review Article

Nervous System of *Periplaneta americana* Cockroach as a Model in Toxinological Studies: A Short Historical and Actual View

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Nervous system of *Periplaneta americana* cockroach is used in a wide range of pharmacological studies, including electrophysiological techniques. This paper presents its role as a preparation in the development of toxinological studies in the following electrophysiological methods: double-oil-gap technique on isolated giant axon, patch-clamp on DUM (dorsal unpaired median) neurons, microelectrode technique *in situ* conditions on axon in connective and DUM neurons in ganglion, and single-fiber oil-gap technique on last abdominal ganglion synapse. At the end the application of cockroach synaptosomal preparation is mentioned.

1. Introduction

The cockroach, especially the *Periplaneta americana* species, is recognized as a very useful model in neurobiological studies [1]. The field of toxinology owes much to the use of various nervous preparations obtained from this insect. *Periplaneta americana* represents an excellent model applied in different pharmacological methods, especially in electrophysiology, which plays a vital part in most of research activity in toxinology. It may be based on using natural and "artificial" preparations, as for example the transfected *Xenopus* oocytes. A wide range of nervous functions have been described on the basis of studies on various parts of cockroach nervous system (Figure 1(a)) and the experiments can be performed on natural models. Biophysical principles of the nervous system function in insects are much the same as in mammals. In both groups of animals similar neurotransmitters can be found, although their distribution varies. Thus the observations made on the cockroach can nearly be applied in vertebrates. On the other hand, some arthropod neurotoxins show high selectivity to insect nervous system and they are considered as potent bioinsecticides. Cockroach model has been largely used for the description of their mode of action. In the following paragraphs, various electrophysiological methods using cockroach preparations will be presented, along with their contribution to the development of toxinology.

2. Giant Axon and Single-Fiber Double-Oil-Gap Method

Cell body and the dendritic tree of the *P. americana* giant interneurons, located in the last abdominal ganglion, have been well identified for a long time. They possess unmyelinated, very long (1.5–2 cm), large diameter (up to 50 μm) axons. The axons, being surrounded by glial Schwann cells, can be isolated manually (Figure 1(b)) under microscope, and their activity can be observed using the double-oil-gap method—a refined electrophysiological technique—Figure 2(a) [2–5]. Such axonal preparation exhibits simple bioelectrical properties; one type of voltage-dependent sodium and potassium (Kdr) channels can be found; they are responsible for generating short (0.5 ms) action potentials. Double-oil-gap technique permits, in current-clamp, to evoke large (up to 100 mV) axonal action potentials (Figure 2(b)(A)), to follow their evolution and to control the resting potential as well as the level of local response. Passive characteristics

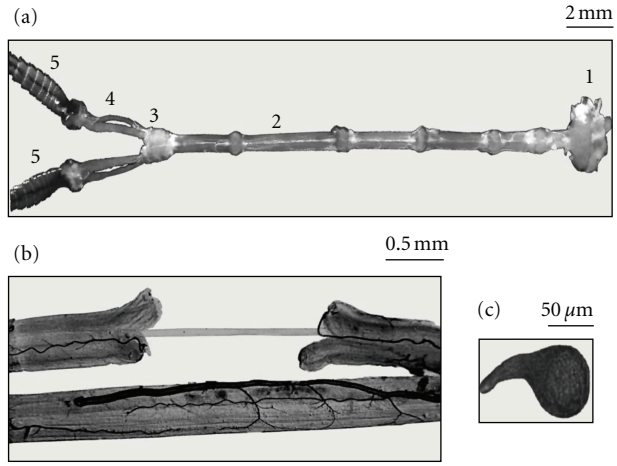

Figure 1: Cockroach (*Periplaneta americana*) nervous system. (a) Isolated abdominal nerve cord with last thoracic (1) ganglion and cercal nerves (4) linked to cerci (5). Giant axon is isolated from one connective between the 4th and the 5th ganglion (2). DUM neurons and cercal nerve-giant interneuron synapses are located in last abdominal ganglion (3). (b) Isolated giant axon dissected from one connective, accompanied by the second connective which protects the axon when the preparation is transferred to experimental chamber; axon diameter ~50 μm. (c) Isolated DUM neuron.

of axonal membrane (resistance and capacitance) can be analyzed using longer (e.g., 5 ms) hyperpolarizing pulses (Figure 2(b)(B)); the application of long depolarizing pulses allows to observe delayed rectification (Figure 2(b)(B)).

In voltage-clamp configuration, depolarizing voltage pulses induce a short, inwardly directed, tetrodotoxin-sensitive sodium current and delayed, noninactivating outward potassium current (Figure 2(d)), most sensitive to 3,4 diaminopyridine [6]; hyperpolarizing pulses are used to record the leak current. At holding potential of −60 mV, about 50% of sodium current is inactivated; this inactivation is completely removed when the HP reaches −80 mV and, in these conditions, a larger current can be observed, similarly to what has been reported by Pichon [2] (Figure 2(e)). Double-oil-gap method has several advantages: (1) the recordings are stable and long-lasting (up to 1 hour), (2) various experimental protocols in current-clamp as well in voltage-clamp can be applied, (3) the quantity of the tested molecules can be very small (e.g., 0.2 mL 10^{-7} M in the case of a highly active substance), (4) it is inexpensive. However, the preparation of an isolated axon is a sophisticated procedure and requires much experience.

The activity of giant axon can also be recorded in *in situ* conditions in the whole nerve cord [7]. In such case, axon is left on its place in nervous chain, stays in contact with others axons and its microenvironment is much less changed compared to isolated axon. After one connective is desheathed, the giant axons become accessible for microelectrodes. Introducing them allows to record the exact axonal resting potential in its *semi*-physiological microenvironment. Extracellular stimulation, even performed from some mm distance, evokes action potentials similar in size and amplitude to action potential recorded from the isolated axon (Figure 2(c)), however, long-pulse stimulation can only accelerate the generation of action potential. The accessibility of the axon *in situ* to the tested molecules is much more limited, however, their effect can be considered more physiological in such conditions.

Double-oil-gap method has been used in toxinological studies for many years. The long history of anti-insect scorpion toxins started with this technique, when in the 1970–80s the toxins purified from North African scorpions (*Androctonus australis* Hector—AaH IT and *Buthus judaicus*—Bj IT) by Professor Eliahu Zlotkin and collaborators from the Hebrew University of Jerusalem, Israel, and Faculté de Médecine, Marseille, France [8], were tested for the first time on cockroach isolated giant axon by electrophysiologist Professor Marcel Pelhate, at Angers University, France [9, 10]. Toxicity tests and biochemical studies, especially by means of binding assays, established the specificity of this toxin, which has been confirmed by electrophysiological experiments. In the subsequent years, the story developed with the application of more and more modern methods and presently many details of arthropod toxins' selectivity to insects are known [11–13]. Their applicability as bioinsecticides is now under consideration [12, 14, 15].

Scorpion toxins, active on sodium channels, are divided into two large groups: *alpha* and *beta* toxins, modifying mainly the channels' inactivation and activation, respectively [12, 13, 16, 17]. Among *alpha* toxins, an *alpha*-like subgroup has been established [18, 19]. The insect-selective toxins, depressant and excitatory, are *beta* toxins. Several toxins related to these groups were tested and their mode of action was determined using the single-fiber oil-gap method. It helped to discriminate between excitatory and depressant groups of toxins. The most distinctive features of axonal bioelectric activity modification induced by excitatory toxins (AaH IT1 from *Androctonus australis* Hector, Bj IT1 from *Buthotus judaicus*—[9, 10, 20]; Bm 32-VI and Bm 33-I from *Buthus martensi*—[21]; are a slight depolarization, a decrease in threshold for action potential generation (Figure 3(a)(A) and (B)), and the repetitive activity instead of a single

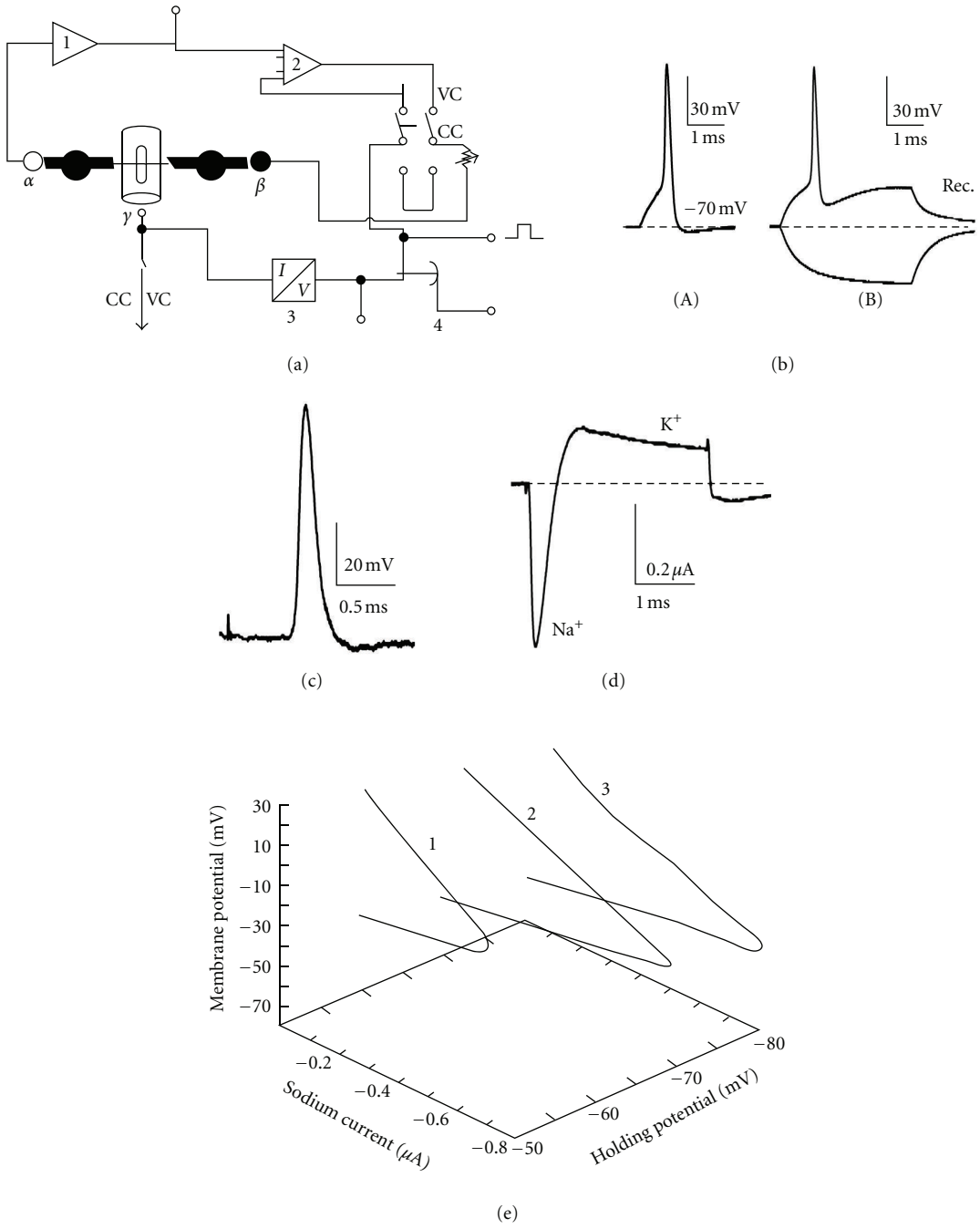

Figure 2: Single-fiber double-oil-gap method on isolated giant axon. (a) Electronic arrangement of technique for current- and voltage-clamp recordings (CC and VC, resp.)—according to [4]. α and β are the lateral (recording and stimulating) electrodes immersed in 180 mM KCl—they have contact with the cut ends of axons in connective and represent intracellular electrodes; γ electrode is plunged in physiological saline (with the tested substances)—it has contact with the extracellular side of the isolated axon. Amplifier 1 is a high-input impedance, negative capacitance amplifier, 2—high-gain differential amplifier, 3—current-to-voltage converter; 4—analogue compensator for leakage and fast and slow capacitive currents. In CC γ electrode is grounded and resistor = 3 MΩ. (b)(A) Action potential evoked by a 0.5 ms depolarizing current pulse. (b)(B) The effect of long symmetrical current pulses: the hyperpolarizing one—used to estimate the passive axonal membrane properties and the depolarizing one—used to observe the membrane rectification (Rec.). (c) Action potential recorded from *in situ* giant axon using microelectrode. (d) Total current recorded under voltage pulse from holding potential −70 mV to −10 mV; K^+—potassium component, Na^+—sodium component. (e) Voltage-dependence of sodium current recorded at various holding potentials: −1 : −60, 2 : −70 and 3 : −80 mV. The points (not shown) for curves are mean values from 5 experiments performed in control conditions. Note that at holding potential of −60 mV, about 50% of Na current is inactivated.

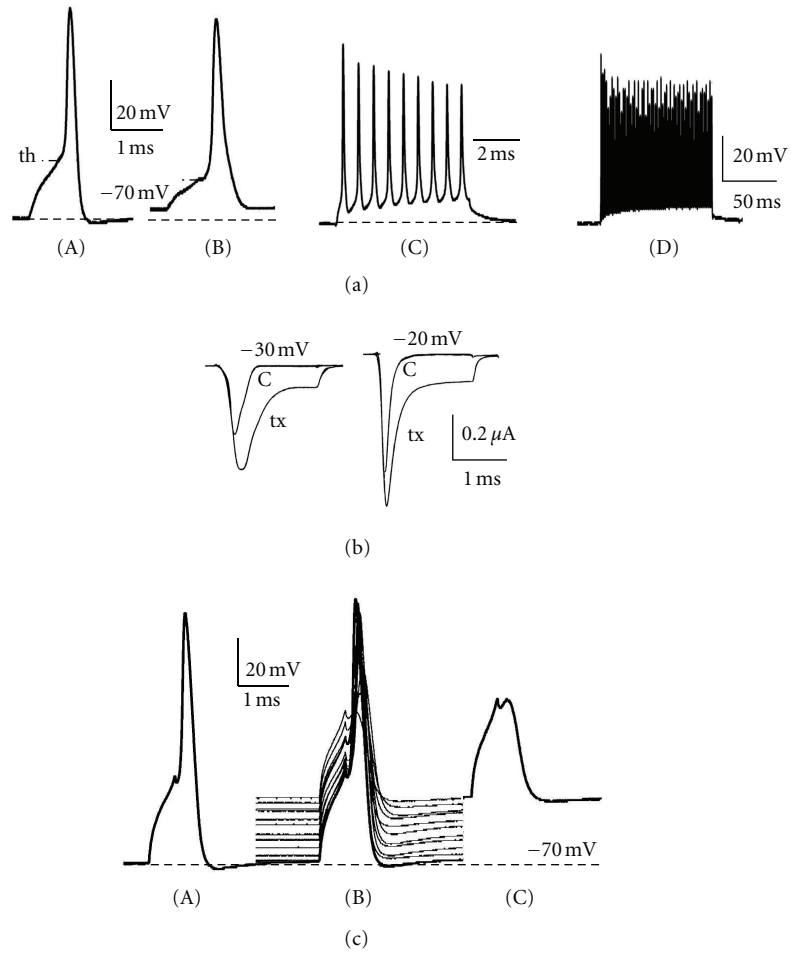

Figure 3: The effects of beta group of scorpion toxins on axonal bioelectrical activity. (a) The effect of Bj-xtrIT—a recombinant excitatory anti-insect toxin from *Buthus judaicus* scorpion: (A) control action potential; (B) action potential recorded from the same axon in 10 min of toxin action (10^{-7} M)—note much lower threshold (th) for action potential generation and a slight membrane depolarization; (C) repetitive activity evoked after artificial repolarization of axonal membrane and stimulation with single, short (0.5 ms) current pulse; (D) long duration, high frequency repetitive activity observed after the artificial membrane hyperpolarization to −80 mV—activity was evoked by a single, short stimulation. (b) Bigger and prolonged sodium current recorded after 10 min of Bj-xtrIT presence (tx, 10^{-7} M) compared to control (c); currents were elicited with voltage pulses from HP = −70 mV to −30 and −20 mV. (c) Axonal activity modified by Lqh IT2 (10^{-6} M)—anti-insect depressant toxin. (A) control action potential and (B) the progressive decrease of its amplitude together with the membrane depolarization induced by toxin; (C) block of action potential generation observed after 15 min of toxin action.

response to current stimulation (Figure 3(a)(C)). The ability of the axon to generate such bursting discharges manifested itself especially when it was artificially repolarized or hyperpolarized (Figure 3(a)(D)). Exalted axonal excitability resulted from the increase in sodium current amplitude at negative membrane potentials and its prolongation observed under voltage pulse (Figure 3(b)). The analysis of sodium current voltage dependence revealed a shift in sodium current voltage dependence towards more negative membrane potentials [20]. In toxicity tests, these toxins caused an immediate contraction paralysis of fly larvae and a quick excitatory "knock-down" effect on locusts [8, 10], which remains consistent with the observations done in electrophysiological experiments.

The name "depressant toxins" comes from the flaccid paralysis they cause in fly larvae. The toxins within this group (Bj IT2—[10]; toxin from *Scorpio maurus palmatus* venom—[22]; Bot IT3 and BoT IT4 from *Buthus occitanus tunetanus*—[23, 24]; BmK ITa and BmK ITb from *Buthus martensi* Karsch—[23]; Lqh IT2—[25] evidently increase sodium permeability at resting potential, inducing relatively fast depolarization and distinct decrease in action potential amplitude (Figure 3(c)(A) and (B)), eventually blocking the conduction (Figure 3(c)(C)). In voltage-clamp experiments, the lowering of peak sodium current was observed, however, along with a development of a constant current, at holding potential equal to axonal resting potential, that is, −60 mV.

There have also been cases of finding toxins of which the effects on axonal electrophysiological activity appear intermediate between those of excitatory and depressant groups, for instance: BcTx1 from East African scorpion *Babycurus centrurimorphus* [26] or BoT IT2, which, moreover, induced

a new, unusual current with slow activation/deactivation kinetics [23, 24, 27]. Bot IT2 is insect-selective; its binding site is similar to that one of AaH IT1 excitatory toxin, but amino acid sequence resembles the ones found in depressant toxins [23, 24]. Similar modification of axonal bioelectrical activity by Bot IT2 has been evidenced using a toxin from the venom of ant, *Paraponera clavata*, also tested on the cockroach isolated axon [28].

The comparative analysis of electrophysiological effects of several excitatory and depressant toxins suggests that the discrimination between these two groups of toxins is not always easy and certainly not possible only on the basis of bioelectrical activity modifications. Parallel biochemical and structural studies are absolutely required. As mentioned above, these two groups of toxins belong to one very large group of *beta* toxins that bind to receptor 4 on sodium channel [13, 14, 17].

Toxin VII from South American scorpion, *Tityus serrulatus*, is a typical *beta* toxin with no selectivity to any group of organisms [29, 30]. Its effect on cockroach axon can also be described as intermediate between those of excitatory and depressant toxins. In the presence of the toxin, resting depolarization was observed jointly with a tendency to repetitive discharges. Sodium current was activated at more negative potential than normally; it was prolonged, but holding current development was slower than in the case of depressant toxins [31].

Scorpion *alpha* toxins bind to the receptor site 3 on sodium channel and inhibit the channel's inactivation [12]. In voltage-clamp experiments, short sodium current is prolonged during depolarizing voltage pulse (Figure 4(b)); potassium current is not modified. In current-clamp, short action potentials are extended and transformed into *plateau* action potentials (Figure 4(a)). Such effects were observed when the first anti-insect toxin LqhαIT from *Leiurus quinquestriatus hebraeus* scorpion venom was tested on cockroach isolated axon [32, 33]. Later, several new anti-insect toxins with similar LqhαIT characteristics were described, as for example BoT IT1 [23, 24].

Alpha toxin (LqhαIT) was also tested on cockroach giant axon in *in situ* conditions. The first observed effect, after the toxin's application, was a repetitive generation of slightly prolonged action potentials in response to short stimulation—such effect has never been observed in the experiments on isolated giant axon (Figure 4(c)). Later on, *plateau* action potential sometimes appeared (Figure 4(d)), but it was never as long in duration as in the case of isolated axon, even after a prolonged application of high toxin concentration. The influence of axonal microenvironment created by glial cells and other axons is a very likely factor in this matter. And it is necessary to consider the fact that the experimental conditions may vary from the physiological ones in a very diverse degree, which in turn may affect significantly the effects of the tested molecules.

Electrophysiological experiments performed on the cockroach axon helped to define a new group of scorpion neurotoxins: the *alpha*-like toxins. Receptor site of these molecules overlaps with receptor site 3 on sodium channel, where *alpha* toxins bind [18, 19]. *Alpha*-like toxins do not express selectivity toward insect or mammalian sodium channels. Pharmacological characteristics of *alpha*-like toxins are similar but not identical with those of *alpha* neurotoxins. They induce *plateau* potentials, but in addition, they depolarize progressively the axonal membrane. They inhibit the inactivation of sodium current, but to a lesser degree than in the case of LqhαIT. Moreover, a tail current appears, which increases when depolarizing pulses are applied repeatedly (every 5 s) and at the same time, the peak sodium current decreases [18, 19].

Along with the science progressing, modern techniques in molecular biology were applied and a "new era" in scorpion anti-insect toxins launched. One of the most noteworthy approaches towards this issue so far have been presented by Professor Michael Gurevitz and Dr. Dalia Gordon from the Department of Molecular Biology and Ecology of Plants, Tel Aviv University, Israel. They and their collaborators isolated the genetic material responsible for the synthesis of toxins from the scorpion venom, they defined the cDNA sequence and developed artificial expression systems [33–36]. New, recombinant toxins LqhαITr [36], Lqh IT2 [37] and Bj-xtrIT [38], tested on the cockroach isolated giant axon, showed much resemblance in their mode of action to the native counterparts. Such studies provided an extremely valuable conclusion: the activity of properly prepared recombinant toxins is the same as that of the native ones. This was a huge step in the field of toxinology. Nowadays, the recombinant toxins are accessible in a greater number than the native molecules and the advent of further mutations is feasible. It is pivotal now to study the molecular basis of anti-insect selectivity, as well as anti-mammalian specificity.

Toxicity tests, binding studies and electrophysiological recordings along with the molecular modeling, gene cloning and the site-directed mutagenesis create an opportunity to investigate the molecular basis of toxins' activity. The experiments performed on *alpha*-like toxins from *Buthus martensi* Karsch using cockroach axon showed that mutation of a single amino acid can change completely the toxin mode of action [39]. A phrase: "a story of one amino acid" may well summarize the long history of multiapproach studies on depressant toxins from *Leiurus quinquestriatus hebraeus.* In the experiments on the isolated axon of the cockroach, it has been shown that the replacement of the amino acid in position 58 from Asn to Asp is able to change completely the toxin's mode of action. At the same time, its affinity to sodium channel target and its toxicity decreased. Conclusion from these studies was as follows: Asn in position 58 plays the mandatory role in the activity of depressant toxins [13, 40].

The isolated axon of the cockroach was also used in the tests on the toxins obtained from the spider venom. In most cases they prolonged the duration of action potential, however, *plateau* action potential could only be generated in the presence of a potassium channel blocker. Sodium current inactivation was inhibited in the presence of the toxin, but never to such a degree as in the case of scorpion *alpha* toxins [41–43]. Postapplication of LqhαIT increased the late sodium current recorded during depolarizing pulse (Stankiewicz, personal observations).

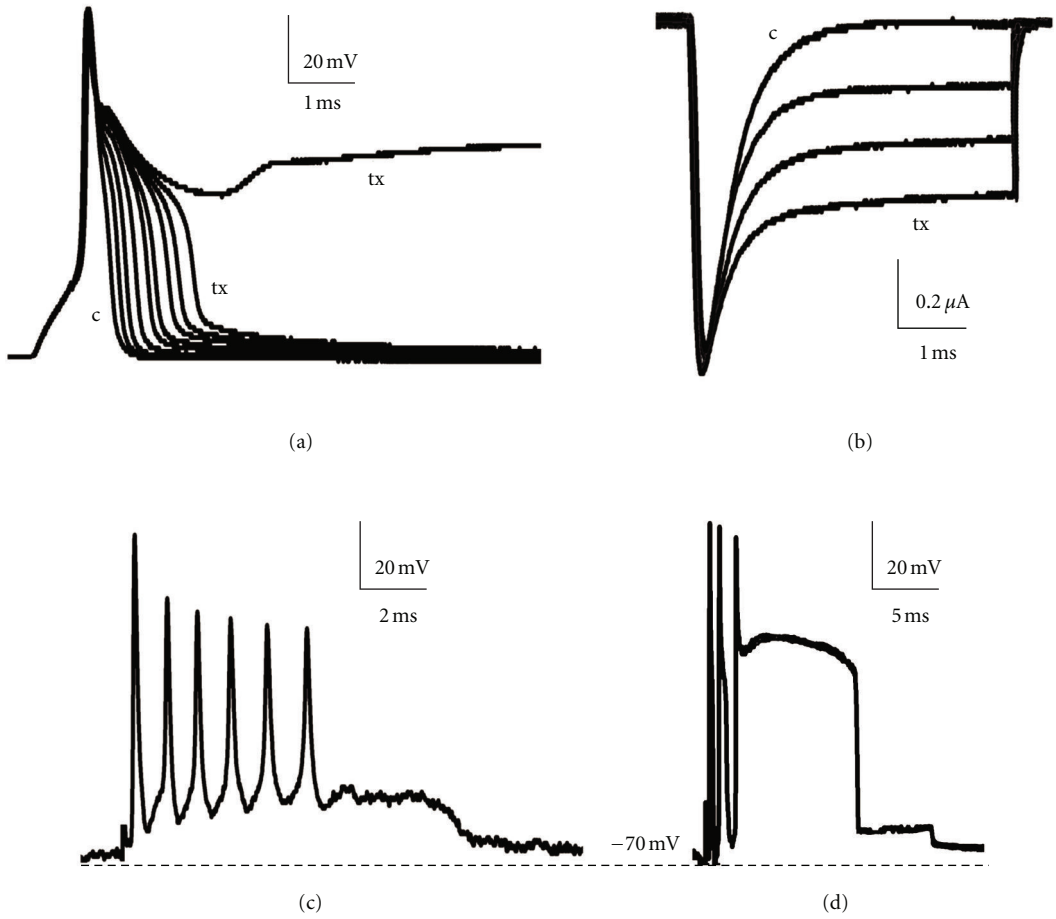

Figure 4: (a) The effect of LqhαIT (10^{-7} M)—recombinant anti-insect *alpha* toxin from *Leiurus quinquestriatus hebraeus* venom on axonal action potential; c—control action potential, tx—progressive increase of action potential duration until *plateau* action potential under toxin. (b) sodium current prolonged until the end of depolarizing pulse in toxin presence (tx). (c) Repetitive discharges induced by LqhαIT (5×10^{-6} M) recorded from the axon in connective (*in situ*), using microelectrode technique; connective was stimulated by extracellular silver electrodes through an isolated unit. (d) *Plateau* action potential generated sometimes by axon *in situ* in toxin presence.

3. DUM Neurons in Patch-Clamp and Microelectrode Techniques

In 1989 and 1990 two articles describing the application of patch-clamp technique in the study on the activity of neurosecretory dorsal unpaired median (DUM—Figure 1(c)) neurons from terminal ganglion of *Periplaneta americana* nerve cord were published [44, 45]. The technique of single DUM cell isolation and patch-clamp recording on it have been developed in Laboratory of Neurophysiology at Angers University in France by Professor Bruno Lapied. DUM neurons possess an endogenous pacemaker activity which depends on a wide range of ionic membrane conductances [46]. Various types of receptors provide a very precise regulation of the neurons' spontaneous activity and neurosecretory function. DUM neurons represent an outstanding model for studies on intracellular processes [47–49] as well as for pharmacological tests [50, 51]. They have also been used in toxinological experiments.

There were several experiments on DUM cells performed simultaneously with observations on an isolated axon. Although DUM neurons represent a much more complex model of bioelectrical activity than the axon, the observations were similar and the conclusions-compatible [25, 52]. Background sodium channels (bNa) in DUM neurons were examined using the patch-clamp cell-attached technique [53]. They appeared to be a new target for LqhαIT toxin, even more sensitive than the classical voltage-dependent Na channels in this preparation. The activity of bNa is limited at membrane potential −50 mV (DUM neuron resting potential) and can be "liberated" under LqhαIT action or at very negative (−90 mV) membrane potential. In the presence of the toxin (10^{-8} M), unclustered, brief single channel openings in control (at −50 mV) were transformed into large, multistep amplitude bursting activity, separated by periods of silence. Open probability of the channels increased by about 20-fold. Such channel activity was well corresponding to the transformation observed in DUM neurons: from regular beating, spontaneous activity to rhythmic bursting [53, 54].

Background sodium channels in DUM neurons are also the target for *beta* toxin (VII) from Brazilian scorpion,

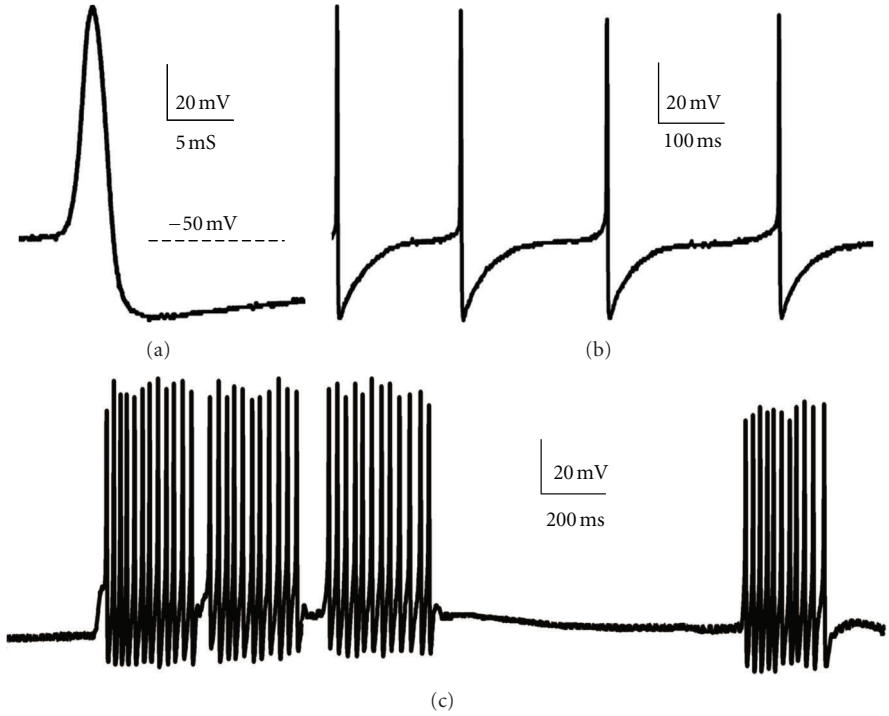

Figure 5: Activity of dorsal unpaired median (DUM) neurons from terminal abdominal ganglion, examined with the microelectrode technique. (a) Single action potential and (b) regular spontaneous activity recorded from the neuron in the ganglion—in *in situ* conditions, in control. (c) Radical change of neuron regular beating discharges into bursting activity in LqhαIT (10^{-6} M) presence.

Tityus serrulatus, venom [55]. In the toxin's presence, single-amplitude openings of the channels were replaced by events with several distinct subconductance current levels. Channel open probability rose by about 50-fold and similarly did the open-time duration; additionally, very long duration events emerged. Classical voltage-dependent sodium channels were also modified in a manner typical for *beta* toxin. The experiments performed with calcium imaging demonstrated the rise in the intracellular calcium concentration in the presence of toxin VII. A very complex study of this phenomenon evidenced the participation of high-voltage activated N-type calcium channels and the activation of noncapacitative calcium entry (NCCE). An important conclusion has been drawn from these studies that the reduction of the activity of NCCE may prove a useful strategy in the development of a drug for antienvenoming therapy [55].

The activity of DUM neurons can also be observed in *in situ* conditions using the microelectrode technique. Terminal abdominal ganglion is separated from the nerve cord, desheated and fixed. Neurons remain in their place in ganglion surrounded by glial cells and other neurons. DUM neurons are recognized by spontaneous action potentials when the microelectrode enters the cell. The pattern of neuronal activity is similar to the recordings obtained on isolated cells, however, the effect of toxins is not exactly the same. The application of *alpha* toxin (LqhαIT, 10^{-5} and 10^{-4} M) never induced *plateau* action potential *in situ*; only transformation from regular firing into bursting activity was observed and the bursts were generated from a level of slight depolarization (Figure 5(c)). In the ganglion, DUM cells are mainly under the influence of glial cells, as well as other neurons; thus, the ionic microenvironment remains partially undisturbed. In order to investigate the toxin's mechanism of action, isolated neurons should be used by all means, however, *in situ* experiments may in some cases reveal more on physiological effect of toxic molecules. The interactions between two neurotoxins observed on isolated and *in situ* DUM cells are also different (Stankiewicz et al., in preparation).

4. Cercal Nerve—Giant Interneuron Synapse and Single-Fiber Oil-Gap Technique

The information about mechanical stimulation arising in the cockroach cercal sensory neurons is transmitted to giant interneurons. Cercal nerves X and XI are connected with giant interneurone dendritic tree by inhibitory and excitatory synapses, respectively, located in the last abdominal ganglion. The activity of these synapses can be observed using the single-fiber oil-gap technique (Figure 6(a)), developed by Professor Jean-Jacques Callec in Rennes University [56, 57], later improved and applied for several years by Professor Bernard Hue from Laboratory of Neurophysiology at Angers University, France [58–60]. This method allows the extracellular recording of spontaneous and evoked excitatory postsynaptic potentials (EPSP) as well as inhibitory postsynaptic potentials (IPSP) [57, 60, 61]. Nerve XI is classified as excitatory and its fibers activate nicotinic receptors at

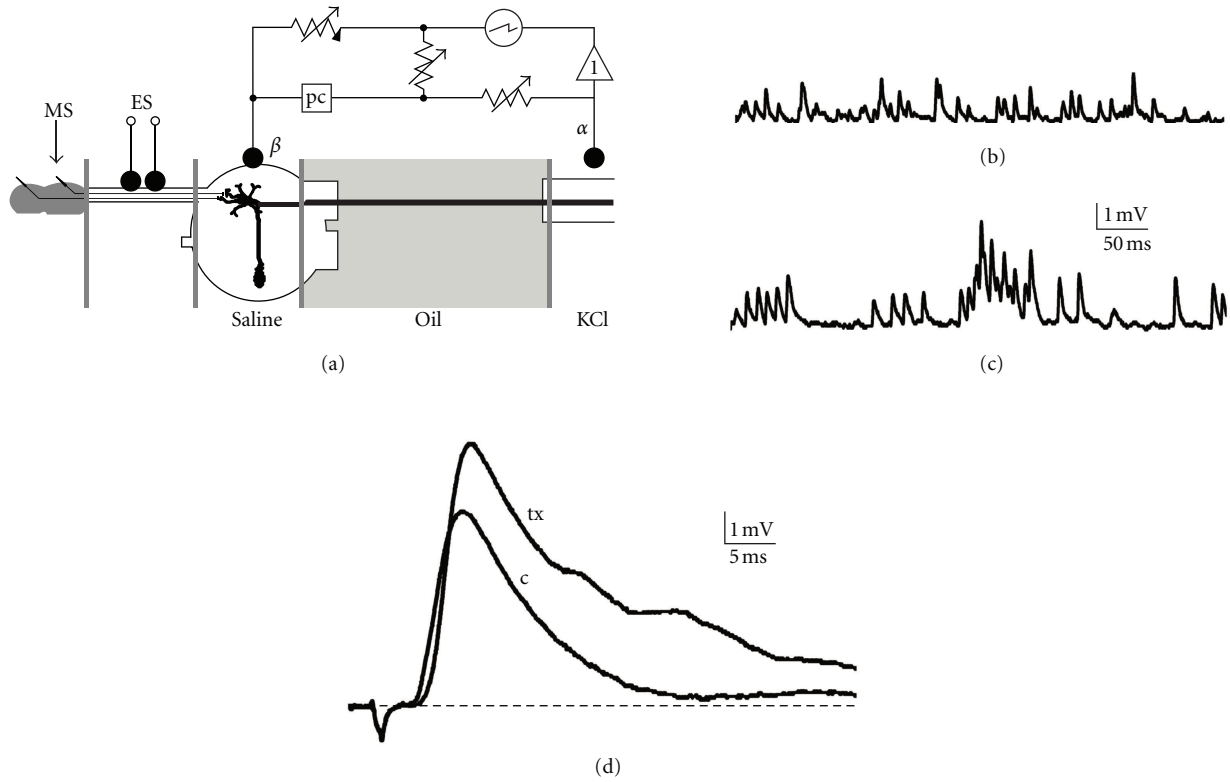

Figure 6: Single-fiber oil-gap method applied for studying synaptic transmission between cercal nerve and giant interneurone. (a) The scheme of electronic circuit used to record postsynaptic events. Recording electrode (α) bathed in isotonic KCl solution is connected with high-input impedance, negative capacitance amplifier (1); extracellular electrode β is in contact with last abdominal ganglion. Wheatstone bridge is connected to recording circuit and it is used to apply polarizing current (pc) through postsynaptic membrane. The desheated ganglion is superfused with saline or test solution. The dissected axon is bathed in paraffin-oil. MS—mechanical stimulation, ES—electrical stimulation applied to cercal nerve (according to [59, 60]). (b) Control conditions—unitary excitatory postsynaptic potentials (uEPSP) recorded as spontaneous activity of preparation and (d) c—control excitatory compound postsynaptic potential (cEPSP) observed as the effect of cercal nerve electrical stimulation. (c) The increased spontaneous activity (uEPSPs) and (d) tx—cEPSPs observed 10 min after LqhαIT (10^{-6} M) application using pneumatic injection in synapse vicinity.

postsynaptic membrane. Unitary excitatory postsynaptic potentials (uEPSP) result from the stimulation of single hair mechanoreceptors covering the cercus; composed postsynaptic potentials (cEPSP) are the effect of cercal nerve XI electrical stimulation. At the presynaptic face of such synapses, muscarinic receptors are present, the role of which is to regulate the acetylcholine releasing by negative feedback [62]. Muscarinic receptors were also found on postsynaptic membrane [63]. Stimulation of nerve X evokes inhibitory postsynaptic potentials (IPSP) via activation of GABA receptors [61]. Single-fiber oil-gap method allows to perform long-term experiments (even up to 12 hours) and well-adjusted perfusion of the ganglion enables to obtain reliable dose-response curves. Using the iontophoretic application of acetylcholine or carbachol to postsynaptic mem-brane vicinity permits to discriminate between pre- and postsynaptic action of the tested drugs. Synaptic preparation from the cockroach represents a remarkably useful model for pharmacological studies, however, obtaining a high-quality stable preparation requires much experience.

Cockroach synaptic model allowed to test the toxins which are active on cholinergic receptors. Snake venom is a well-known source for them. *Alpha*-bungarotoxins and k-bungarotoxins completely blocked uEPSP and cEPSP at concentration of 10^{-7} M. All performed experimental protocols indicated that the toxins block neuronal postsynaptic nicotinic receptors and α-bungarotoxin was more effective [64, 65]. Scorpion *alpha* toxin LqhαIT induced a substantial increase in the postsynaptic spontaneous activity, that is, in the frequency and the amplitude of uEPSP (Figures 6(b) and 6(c)), as well as in the amplitude and duration of cEPSP (Figure 6(d)). Higher presynaptic activity results in the increased releasing of acetylcholine, however, it activates the negative feedback via presynaptic muscarinic receptors in turn and after several minutes of the toxin's action, a decrease in postsynaptic events can be observed (Stankiewicz, personal observations).

The experiments performed using the single-fiber oil-gap method with the scolopendra, *Scolopendra* sp., venom determined its components which induced the depolarization of the cockroach postsynaptic membrane and the decrease in EPSP amplitude. Such effect was limited after pretreatment with atropine. Along with the studies performed on *Drosophila* muscarinic receptors (Dm1) expressed

in *Xenopus* oocytes, it was evidenced that the venom of scolopendra comprises a component acting as agonist on insect muscarinic receptors [66].

Lastly, the experiments performed on cockroach synaptic preparation contributed to studies which allowed to explain the mechanism of synergistic interaction between chemical neurotoxins: pyrethroids (permethrin) and carbamates (propoxur) [67]. The conclusions from these studies are important for practical implementation in the field of crop protection.

5. Synaptosomal Preparation from Cockroach Nerve Cord

The nerve cords of cockroach (*Periplaneta americana*) have also been used to prepare synaptosomes, which are functional vesicles containing the nervous terminals. The synaptosomal preparation is easy to obtain and has been applied in several pharmacological tests. As illustration, binding assays of many radiolabeled toxins have been successfully characterized with synaptosomes [24, 42, 43, 68]. In addition, studies of photoaffinity labeling using ^{125}I TsVII as a ligand in synaptosomes of nerve cord from cockroach indicated for the first time the molecular weight of the scorpion toxin receptor from the insect nervous system which was suggested to be associated with voltage sensitive Na^+ channels [68]. More recently this preparation was also successful applied in binding studies involving radiolabelled spider toxins acting in sodium channels (De Lima, in preparation). Results obtained from electrophysiological experiments are often completed using synaptosomal preparation coming from cockroach nervous system.

6. Summary

Nervous system of the cockroach (*Periplaneta americana*) can be recognized as a remarkably useful model preparation in multiple electrophysiological techniques, which allows to perform pharmacological tests on diverse levels of nervous system organization. Confronting the results obtained reveals that a toxin may affect the activity of the same nervous structure with diverse effects, depending on the experimental conditions and this conclusion should be taken into account prior to any definite statement concerning the mode of action of any toxin.

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Review Article

Ion Channels and Zinc: Mechanisms of Neurotoxicity and Neurodegeneration

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Ionotropic glutamate receptors, such as NMDA, AMPA and kainate receptors, are ligand-gated ion channels that mediate much of the excitatory neurotransmission in the brain. Not only do these receptors bind glutamate, but they are also regulated by and facilitate the postsynaptic uptake of the trace metal zinc. This paper discusses the role of the excitotoxic influx and accumulation of zinc, the mechanisms responsible for its cytotoxicity, and a number of disorders of the central nervous system that have been linked to these neuronal ion channels and zinc toxicity including ischemic brain injury, traumatic brain injury, and epilepsy.

1. Introduction

Although zinc is clearly an essential trace element that is required for the function of hundreds of enzymes and DNA-binding transcription factors, excessive zinc has long been implicated in processes leading to cellular damage. In the central nervous system large amounts of zinc can enter postsynaptic neurons through a variety of ion channels including glutamate receptors and voltage-gated calcium channels. This paper will first discuss the cellular pools of zinc, the mechanisms that appear to be responsible for the accumulation of neuronal zinc, and then outline the current hypotheses about how excess zinc exploits ion channels and other mechanisms to produce neurotoxicity in the hippocampus, amygdala, and cortex under pathological conditions. With these mechanisms in mind we will then discuss relevant clinical situations such as traumatic brain injury, ischemic injury (stroke), and epilepsy, where excess zinc accumulation can lead to neurodegeneration.

2. Neurotoxic Zinc: Cellular Sources and Routes of Entry

2.1. Sources of Neurotoxic Zinc. While a majority of zinc in the central nervous system (CNS) is tightly bound to zinc-dependent enzymes and other proteins, approximately 10% is "free" or "chelatable" zinc which is not associated with proteins or aminoacid ligands. Under pathological conditions, free zinc appears to participate in the neurotoxic accumulation of zinc in neurons. In normal neurons, free zinc is predominately localized to the presynaptic vesicles of glutamatergic neurons [1, 2]. Free zinc has also been colocalized to GABA and glycine containing murine neurons [3]. Regions rich in vesicular free zinc include the mossy fibers of the hippocampus, the amygdala, and the olfactory bulb. "Zincergic" neurons are also abundant in the cortex [4].

In addition to the large pool of vesicular zinc, there is clear evidence for additional intracellular pools of zinc that can be liberated to form free zinc. The strongest case for the presence of nonvesicular pools of free zinc comes from a report showing that free zinc accumulates after seizure activity in animals that lack vesicular zinc [5]. This work measured free zinc accumulation in the hippocampal neurons of ZnT3-null mice that lack the ability to pump zinc into synaptic vesicles. While this work did not definitively determine the source of the non-vesicular free zinc, the surprising finding that these animals exhibited accumulation of free zinc in damaged neurons after kainate-induced seizures began the hunt for alternative pools of free zinc that may participate in neurotoxicity. Subsequently, others

have identified a mitochondrial pool of zinc that can be both influenced by the amount of intracellular zinc as well as contribute to it [6]. There is also now evidence that protein bound zinc can be mobilized to form a free zinc pool under oxidative conditions [7].

2.2. Routes of Neurotoxic Zinc Entry. Upon neuronal excitation, vesicular zinc is released into the synaptic cleft. Under normal conditions, the primary function of the zinc from synaptic vesicles appears to be the modulation of both ionotropic and metabotropic post-synaptic receptors through zinc-specific allosteric binding sites. For example, zinc inhibits $GABA_A$ receptors, reducing their inhibitory action [8, 9]. The effect of zinc on excitatory glutamate receptors is complex. Not only can zinc act as an inhibitory neuromodulator of glutamate release [10], but it was initially thought to inhibit activity of NMDA glutamate receptors [9, 11]. However, there are reports of biphasic and cell type-specific zinc regulation of both NMDA and AMPA/kainate glutamate receptors [12–15]. Additionally, zinc can potentiate glycine-mediated currents [16] and regulate voltage-gated calcium channels [17] as well as potassium, sodium, and chloride channels [18].

However, under pathological conditions, excess free zinc is released from synaptic and other free zinc pools. As excess zinc floods the synaptic cleft, it exploits a variety of receptors and channels to gain entry into post-synaptic neurons. There appear to be at least four different routes of entry. First, AMPA/kainate glutamate receptors have been identified as the primary route of entry for zinc into post-synaptic neurons [19]. Zinc also exploits NMDA glutamate receptors to gain entry into neurons. Both of these glutamate binding receptors transport calcium as well as zinc into post-synaptic neurons. The third route of entry for toxic levels of zinc is voltage-gated calcium channels [19–21]. Finally, excess zinc appears to enter neurons via a transporter-mediated exchange with intracellular sodium. While the presence of a putative Na^+/Zn^{+2} exchanger has been hypothesized, it is also probable that when excess zinc is released from presynaptic neurons, it can replace calcium and gain entry into neurons via Na^+/Ca^{+2} exchange proteins [20].

3. Mechanisms of Zinc Neurotoxicity

Damage to the CNS induced by seizure, trauma, or ischemia can all result in the accumulation of zinc from vesicular and non-vesicular zinc pools. While it is clear that the excess zinc that is detected in these and other pathological conditions is neurotoxic, there is considerable debate about the cause of the toxicity. There are three main hypotheses that are currently being explored, namely, that excess zinc: causes excitotoxicity, induces oxidative stress, and impairs the generation of cellular energy. In fact, as the next several sections will show, there is evidence for each of these mechanisms, suggesting that they are not mutually exclusive, and that all three actions of zinc may be acting synergistically to cause neuronal damage and death.

3.1. Excitotoxicity. The influx of excess zinc into neurons has been shown to result in excitotoxic damage to post-synaptic neurons. This is in part mediated by the action of zinc on glutamate receptors and other ion channels. For example, when large amounts of glutamate and zinc are released together from pre-synaptic neurons, there is an acute block of NMDA glutamate receptors as zinc binds to allosteric sites on these ion channels. However, this acute downregulation is followed by a Src family kinase-mediated upregulation of NMDA receptor activity that appears to participate in neuronal damage and cytotoxicity [12].

3.2. Oxidative Stress. Excess zinc has been implicated in the generation of oxidative stress by free radicals and reactive oxygen species (ROS) leading to neuronal damage and death. In fact, it was recently hypothesized that the combination of age-related oxidation and resulting zinc accumulation could act synergistically to promote aging-related neuronal damage and neurodegeneration [22]. In support of this hypothesis, treatment of mouse cortical neuron cultures with 30–35 μM zinc for 24 hours triggered processes eventually leading to neuronal, but not glial, death [23] that was accompanied by membrane lipid peroxidation. Interestingly, while the neurotrophins NT-3, NT-4/5 and brain-derived neurotrophic factor (BDNF) were unable to prevent the zinc-induced neurotoxicity, the water soluble form of vitamin E, trolox, almost completely prevented the neuronal death associated with excess zinc in these cultures [23, 24]. Zinc toxicity, both as a result of zinc applied directly to neuronal cultures and after kainate application, was also prevented by the addition of glutathione [25]. Together, these data suggest that much of the neuronal damage was the result of free radical generation.

It should be noted that the neuronal death was accompanied by classical features of apoptosis including internucleosomal DNA fragmentation, as well as other characteristics commonly associated with necrosis such as swelling of intracellular organelles [23, 24]. While the authors of this early report suggest that zinc may trigger necrosis, subsequent work has shown that neuronal apoptosis is complex and frequently cannot be categorized into strictly apoptosis or necrosis, but is probably best described as a continuum, with characteristics that include those classically associated with both apoptosis and necrosis [26].

Clearly the next question is *how* excess zinc leads to the production of ROS. Early evidence suggested a role for zinc in the regulation of neuronal protein kinase C (PKC) [27]. While this initial report linked the zinc-regulation of PKC to oxidative damage, it was subsequent work that led to the elucidation of the mechanism responsible for the PKC-dependent generation of ROS in zinc-loaded neurons. Namely, it appears that zinc-stimulated PKC enhances the expression of the enzyme NADPH oxidase. Zinc also facilitates the translocation of the NADPH oxidase subunits $p47^{PHOX}$ and $p67^{PHOX}$ to the neuronal membrane, where they participate in the generation of ROS [28]. Zinc-stimulated NADPH oxidase may also stimulate the activation of poly (SDP-ribose) polymerase (PARP), which can trigger

apoptotic processes [29]. While it is clear that these may not be the only mechanisms responsible for the generation of ROS in zinc-loaded cells, the work does shed light on the role of oxidation in zinc toxicity.

3.3. Impaired Energy Production. While early work showed that nanomolar concentrations of zinc were capable of significantly inhibiting the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), our current understanding of the role of zinc in neuronal glycolysis is that excess zinc rapidly reduces cellular levels of nicotinamideadenine dinucleotide (NAD^+) [30–32]. This reduction in NAD^+ is not only responsible for the reduced activity of GAPDH and the subsequent accumulation of dihydroxyacetone phosphate (DHAP) and fructose 1,6 bisphosphate, but also leads to a reduction in ATP levels and subsequent neuronal death [30]. Reductions in NAD^+ would be expected to reduce cellular ATP production not only because it inhibits the activity of glycolytic enzymes, but also because of the resulting inhibition of mitochondrial respiratory enzymes. In fact, several studies have reported reductions in the NAD^+ dependent enzymes alpha-ketoglutarate dehydrogenase and isocitrate dehydrogenase as well as other mitochondrial enzymes including succinate dehydrogenase and aconitase [33, 34].

Other mechanisms leading to zinc-induced mitochondrial dysfunction including the RAS/ERK/MAPK signaling pathway [35], where ERK 1/2 is likely activated via zinc induction of the immediate early gene *erg-1* [36], and MAPK signaling leads to mitochondrial hyperpolarization [35]. Furthermore, mitochondrial trafficking, which has been hypothesized to play a role in neurodegeneration, is impaired by excess zinc, potentially by a phosphatidylinositol 3-kinase-mediated mechanism [37]. Not only can zinc act as an independent modulator of mitochondrial function, it also appears to act synergistically with other agents such as kainate to induce mitochondrial dysfunction and neuronal death [38, 39]. This appears to be facilitated by the mitochondrial uptake of zinc, followed by the release of proapoptotic mitochondrial proteins such as cytochrome c and the apoptosis induction factor, AIF [40].

4. Zinc Toxicity in Neurological Disease and Disorders

The proposed mechanisms associated with zinc neurotoxicity have been implicated in a large number of neurodegenerative disorders including amyolateral sclerosis, Alzheimer's disease, and Parkinson's disease. The strongest links, however, between the excess zinc release and uptake via ion channels have been observed in models of ischemic brain injury, traumatic brain injury, and seizure disorders.

4.1. Zinc and Ischemic Brain Injury. Although its role is under active investigation, zinc dysregulation has been implicated in mechanisms leading to neuronal damage and death after ischemic brain injury. Tønder et al. first suggested a role of zinc toxicity in the neuronal death associated with global ischemia. In this early report, when adult rats were subjected to global brain ischemia there was a decrease in free zinc associated with mossy fiber layers of the hippocampus and an increase in the dentate hilar region of degenerating neurons [41]. In another model of stroke, transient forebrain ischemia, zinc left pre-synaptic terminals and accumulated in post-synaptic neurons. This translocation was associated with degeneration of neurons in the hippocampus, cerebral cortex, thalamus, striatum, and amygdala. An intracerebral injection with the membrane impermeant zinc chelator Ca-EDTA prior to ischemia was neuroprotective and significantly decreased neuronal death [42]. Administration of Ca-EDTA 30 min prior to mild focal ischemia reduced infarct size 3 days after injury. However, the infarct reduction was lost when rats were euthanized 14 days later, even when Ca-EDTA was continuously perfused [43].

Aspirin is an anti-inflammatory drug commonly used for secondary prevention after stroke and is protective against NMDA-induced and zinc-induced neurotoxicity. The application of 3–10 mM aspirin to cortical neurons exposed to 300 μM zinc for 30 min prevented neuronal degeneration but not oxidative stress or apoptosis. It was shown that aspirin prevented zinc entry into neurons by inhibiting voltage-gated calcium channels, which is a main route for zinc entry into neurons [44].

4.2. Zinc and Traumatic Brain Injury. There is also a relationship between accumulation of free zinc and neuronal degeneration following traumatic brain injury. In one study, adult rats and neonatal pups were given a unilateral cortical stab wound. Four weeks following injury, adult rats, but not neonatal pups, showed increased TSQ staining of free zinc in and around the site of injury, suggesting that the accumulation of free zinc is responsible for neuronal death [45]. A TBI model of mechanical cortical trauma in rats resulted in loss of zinc from pre-synaptic vesicles and movement into post-synaptic neurons accompanied by neuronal death in the hilus, dentate gyrus, and CA1 regions of the hippocampus [46]. It was further demonstrated that rats given a mild or moderate TBI by a fluid percussion injury showed that injured neurons stained with Fluoro-Jade in the hippocampal regions of CA1, CA3, and dentate gyrus 4 hours and 24 hours after injury contained high levels of free zinc as detected by the zinc indicator Newport Green. This is an important finding because it suggests that an increase in free zinc after brain injury is neurotoxic and causes neuronal death in the hippocampus [47].

Additional evidence that high levels of free zinc are involved in neuronal degeneration after brain injury comes from experiments demonstrating that injections of the extracellular zinc chelator Ca-EDTA before TBI provided a neuroprotective effect by reducing cell death in the hippocampus [46]. Pretreatment with Ca-EDTA before a TBI-induced model of fluid percussion injury reduced apoptotic cell death measured by TUNEL labeling while also upregulating the expression of neuroprotective genes [48]. However, rats that were given a TBI and Ca-EDTA treatment did not have improved learning and memory performance in the Morris

Water Maze test two weeks after TBI. Although zinc chelation provides short-term histological benefits, it does not appear to improve long-term functional outcomes [49].

Interestingly, recent evidence has suggested that the source of the zinc that accumulates after TBI in mice is not exclusively vesicular and has led researcher to question some of the previous assumptions about the role of zinc in brain injury. ZnT3-knockout mice that lack vesicular zinc showed an increase in injured neurons and apoptotic cells compared to wild-type (WT) control mice 24 hours after injury. After chemical blocking of vesicular zinc in post-TBI ZnT3 KO and WT mice, damage was unchanged in ZnT3 KO mice, while the numbers of apoptotic cells increased in WT mice with levels comparable to ZnT3 KO mice. This new evidence suggests that free vesicular zinc may actually play a neuroprotective role after TBI [50]. Clearly more work will be needed to understand the role of zinc, glutamate receptors, calcium channels, and other mechanisms after traumatic brain injury. In light of these new data it is interesting to note that administration of zinc appears to have a positive effect on outcomes after TBI. In one clinical trial, zinc supplementation improved neurological scores following TBI [51]. More recent work in a rat model of TBI showed that zinc can provide behavioral resiliency to TBI when given 4 weeks prior to injury and resulting in improved cognition and reduced depression- and anxiety-like behaviors [52]. The mechanisms responsible for the robust effect of zinc in this model are currently under investigation ZnT3.

4.3. Zinc and Epilepsy. Zinc dysregulation and homeostasis has been suggested to play a role in the development of seizures. In an epilepsy mouse model, zinc levels were significantly lower in the hippocampus when compared to control mouse strains [53]. There is also evidence that zinc translocates from pre-synaptic boutons into post-synaptic neurons, possibly causing neuronal degeneration after seizure [54].

As discussed previously, synaptic zinc acts as a neuromodulator and regulates the activity of a variety of post-synaptic receptors including NMDA, $GABA_A$, and AMPA receptors, which then determine neuronal excitability [55]. As a result of its ability to act as a neuromodulator, zinc has been implicated as a proconvulsant [56] and anticonvulsant [57]. Studies that support the proconvulsant theory showed that seizures were induced by an injection of zinc sulfate in rabbits [56]. It has also been shown that zinc chloride enhances kainate neurotoxicity in the hippocampus. Interestingly, CNQX, an NMDA antagonist, helped to prevent neuronal damage [58]. During kindling-induced seizures in which a stimulus was applied for ten times a day for two days, and the zinc chelator diethildythiocarbmate (DEDTC) was injected before each stimulus, duration of behavioral seizures and electrical discharges were decreased [59].

Despite this work, most data support the idea of zinc as an anticonvulsant. Increased susceptibility to kainate-induced seizures was observed in both ZnT3 KO mice lacking vesicular zinc [55] and in mice fed a zinc deficient diet for 4 weeks [60]. After kainate treatment, hippocampal extracellular fluid in zinc deficient mice had increased levels of glutamate and decreased levels of GABA compared to control animals [60]. Furthermore, in a mouse model of epilepsy, seizure susceptibility was increased by zinc deficiency and reduced by zinc supplementation [61]. Chelation studies further support the anticonvulsant theory in that membrane permeable and membrane impermeable zinc chelators have little effect on seizure activity in the CA3 region of the hippocampus [62].

Since most studies suggest that zinc plays an anticonvulsant role in seizures, it is important to look at the possible role of zinc supplementation as a therapeutic agent in the treatment of seizure. The administration of zinc directly into the dentate gyrus of the hippocampus delayed behavioral seizures during electrical stimulation in adult Wistar rats [63]. Intraperitoneal injections with a medium dose of zinc (3 mg/kg) and with the antiepileptic drug valproic acid either alone or in combination reduced the severity of pilocarpine-induced seizures. In contrast, high doses (60 mg/kg) of zinc exacerbated the severity of seizures highlighting the dose-dependent effect of zinc [64].

5. Conclusion

Neuronal ion channels and receptors and zinc toxicity appear to play a role in a number of neurological disorders and diseases. Gaining access to neurons through these receptors and channels, excess zinc leads to excitoxicity, oxidative stress, and impairment of neuronal energy production, all of which not only damage neurons but also lead to neuronal death. Future work will be needed to develop strategies to block zinc-mediated damage and prevent poor outcomes associated with stroke, traumatic brain injury, and epilepsy as well as other neurodegenerative disorders that may respond to therapies designed to modulate ion channels and zinc.

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