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Abstract

Acid-sensing ion channels (ASICs) are voltage-independent proton-gated cation channels that are largely expressed in the nervous system as well as in some non-neuronal tissues. In rodents, six different isoforms (ASIC1a, 1b, 2a, 2b, 3 and 4) can associate into homo- or hetero-trimers to form a functional channel. Specific peptide toxins targeting ASIC channels have been isolated from the venoms of spider (PcTx1), sea anemone (APETx2) and snakes (MitTx and mambalgins). They exhibit different, and sometimes partially overlapping, pharmacological profiles and are usually blockers of ASIC channels, except for MitTx which is a potent activator. This review focuses on the use of these peptides to explore the structure-function relationships, the physiological and the pathophysiological roles of ASIC channels, illustrating at the same time the therapeutic potential of these natural compounds.

1. Introduction

Under the double evolutionary pressure to select the best hunting and self-defense strategies (against predators or competitors), venomous animals have engineered innovative and unique protein structural motifs that can target a variety of receptors and ion channels with high affinity and specificity. Since more than 40 years, venoms of species like spiders, scorpions, sea anemones, snakes and cone snails have provided a rich pharmacopoeia. With therapeutic motivations, scientists first identified the toxic components, called toxins, responsible for severe animal envenomations (Nicholson et al. 1998; Miranda et al. 1970; Servent et al. 2000; Karlsson 1979; Renaud et al. 1986; Chang 1979; Olivera et al. 1985). They further identified the molecular targets of venom toxins to understand their mode of

action, and showed that some venom peptides were also of high interest for basic science (McCleary and Kini 2013; Menez 1998; Twede et al. 2009; Han et al. 2008; Harvey 2001). A number of venom peptides have been successfully exploited for medical purposes too. Angiotensin converting enzyme (ACE) inhibitor from the venom of the Brazilian arrowhead viper *Bothrops jararaca* led for instance to a potent anti-hypertensive drug (Captopril), which is one of the most prescribed drugs in the world (Koh and Kini 2012; Ferreira et al. 1970).

Animal toxins were key pharmacological tools in the ion channel field to study structure-function, gating mechanism and tissue localization of many channels (Dutertre and Lewis 2010). Some of these peptides even lead to clinical development and venom-based drugs, like in recent years ziconotide (SNX-111, Prialt), an inhibitor of neuronal voltage-gated Ca^{2+} channels isolated from *Conus magus*, for patients with intractable pain who do not respond to other drugs (Schmidtko et al. 2010; Miljanich 2004).

Acid-Sensing Ion Channels (ASICs) were first identified in the laboratory of Michel Lazdunski about 15 years ago (Waldmann, Champigny et al. 1997). Six protein isoforms are coded by four genes in rodents: ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3 and ASIC4 (Garcia-Anoveros et al. 1997; Waldmann, Bassilana et al. 1997; Waldmann, Champigny et al. 1997; Chen et al. 1998; Bassler et al. 2001; Price et al. 1996; Waldmann et al. 1996; Lingueglia et al. 1997; Akopian et al. 2000; Gründer et al. 2000), that associate in homo- or heterotrimers (Jasti et al. 2007). They are activated by extracellular acidic pH to mediate a sodium-selective, amiloride-sensitive current. Interestingly, the human ASIC3 channels have been recently shown to be sensitive to both acidic and alkaline pH in certain conditions (Delaunay et al. 2012). The ASIC2b and ASIC4 subunits do not form functional homomeric proton-gated channels by themselves, but ASIC2b can associate with other ASIC subunits to confer new properties on the heteromeric channels. The role of ASIC4 is still not clear. ASICs are largely expressed in the central and peripheral nervous systems, primarily found in neurons. ASIC1a and ASIC2 (both variants a and b) are present throughout the central and peripheral nervous system while ASIC1b and ASIC3 are primarily found in sensory neurons of the trigeminal, vagal, and dorsal root ganglia that also expressed all the other isoforms. Peripheral tissue acidosis associated with pain occurs in situations like inflammation, ischemia, fractures, haematomas, tumors, wounds or after surgical procedures. ASIC channels are of particular interest since they are highly sensitive to moderate acidifications. They are for instance 10-fold more sensitive than TRPV1, another ion channel activated by protons, capsaicin and heat in sensory neurons. ASIC channels can generate sustained depolarizing currents upon prolonges tissue acidification compatible with the detection of non-adapting

pain. In central nervous system (CNS), ASIC channels have been involved in several pathological conditions like ischemia, seizures or neuroinflammation where extracellular acidification occurs. A role in synaptic physiology has also been suggested, which could be relevant of the acidification of the synaptic cleft upon neuronal activity after the release of the acidic content of synaptic vesicles (Miesenbock et al. 1998; Ozkan and Ueda 1998). ASIC currents and/or transcripts have also been observed in glia, smooth muscle cells, lung epithelial cells, immune cells, urothelial cells, adipose cells, joint cells and osteoclasts, indicating that ASICs likely play a role in non-neuronal cells as well (for reviews see (Noël et al. 2010; Deval et al. 2010; Sluka et al. 2009; Gründer and Chen 2010; Wu et al. 2012; Li and Xu 2011)[Ba1]).

2. Venom toxins targeting ASIC channels

2.1. Four toxins with different structures

To date, four compounds targeting ASIC channels were purified from venoms of various origins. The spider peptide PcTx1, the sea anemone peptide APETx2, the snake peptides mambalgins and the snake dimeric protein MitTx.

PcTx1 was the first high-affinity inhibitor (IC_{50} ~ 1 nM) described for ASIC channels. This 40 amino-acid peptide has been isolated as a minor component (less than 1% of protein content) of the South American tarantula *Psalmopoeus cambridgei* venom (Escoubas et al. 2000). Its solution structure was first solved by NMR in 2003 (PDB code 1LMM, (Escoubas et al. 2003)) and then refined to a more precise model in 2011 (PDB code 2KNI, (Saez et al. 2011)). It consists of a three-stranded anti parallel β-sheet, defining three loops and a compact disulfide bonded core (Fig. 1A, B). PcTx1 is folded according to the Inhibitor Cystine Knot (ICK) motif, also found in other spider and cone snail toxins acting on voltage-gated ionic channels (Pallaghy et al. 1994; Craik et al. 2001). This cystine knot confers a resistance to extreme pH, organic solvents, and high temperatures. This structure is also resistant to proteases, which could be an advantage in regard to galenic and administration routes for therapeutics. Despite this global structural homology with other ICK peptides, PcTx1 has no more than 28% sequence identity with known spider toxins (Escoubas et al. 2000).

The sea anemone peptide APETx2 was the second ASIC-targeting peptide discovered in 2004, as a minor (2%) constituent in the venom of the sea anemone *Anthopleura elegantissima* (Diochot et al. 2004). It is a peptide of 42 amino acids crosslinked by three

disulfide bridges (Fig. 1A). The structure of APETx2 is more closely related to that of K^+ channel modulators. APETx2 displays 64% sequence identity with APETx1, a blocker of HERG K^+ channel, 34% sequence identity with BDS peptides, which block the K_V3 voltage-gated potassium channel, and 25-29% sequence identity with AP-A, AP-B, AP-C toxins known to activate voltage-gated sodium channels (Na_V) (Yeung et al. 2005; Diochot et al. 1998; Diochot et al. 2003; Castaneda and Harvey 2009; Bruhn et al. 2001; Norton 1978). The APETx2 structure was determined by two-dimensional ^{1}H-NMR using the native toxin (Chagot et al. 2005) (Fig. 1B). It consists of a compact disulfide-bonded core composed of a four stranded β-sheet connected by three disulfide bonds, from which a loop (15-27) and the N- and C-termini emerge. It belongs to the disulfide-rich all-β structural family with a fold typical of the Defensin family of peptides (Torres and Kuchel 2004), which also includes APETx1, BDS peptides (Kv blockers), AP and ATX toxins (Nav activators).

MitTx was identified in 2011 from the venom of the Texas coral snake *Micrurus tener tener* (Bohlen et al. 2011). MitTx has a β-bungarotoxin-like structure with two subunits, a MitTx-α subunit consisting of a 60 amino-acid Kunitz type peptide, and a MitTx-β subunit, which is a 120 amino-acid phospholipase A2-like protein (Fig. 1A, B). Both subunits are involved in a 1:1 interaction (Kd ~ 12 nM), but this interaction is non-covalent, unlike the β-bungarotoxins that are linked by disulfide bond.

We have identified in 2012 two 57 amino-acid ASIC-targeting peptides from the African black mamba *Dendroaspis polylepis polylepis* venom, which only differ by one residue (at position 4) and have been called mambalgin-1 and mambalgin-2 (Diochot et al. 2012). An ortholog peptide that differs by one amino-acid at position 23 has also been recently identified in the green mamba *Dendroaspis angusticeps* venom and has been called mambalgin-3 (H. Schweitz and S. Diochot, personal communication) (Fig. 1A, access number #xxx[Ba2]). Mambalgins belong to the family of three finger toxins (3FTxs) but define a new sub-family with no more than 50% sequence identities with other snake 3FTxs (Fig 2). A model of their 3D-structure show the typical three finger fold, with three loops emerging from a core stabilized by four disulfide bonds, but mambalgins have shorter loop I and loop III than other 3FTxs, with only one finger (loop II) protruding from the core (Fig. 1B). They are related to the "non-conventional toxins" also referred as "weak toxins" such as CM-2a, CM-3 and CM-1b, characterized by a low toxicity (LD_{50} varying from 5 to 80mg/kg) (Joubert and Taljaard 1980; Utkin and Osipov 2007) compared to the potent α-neurotoxins (LD_{50} from 0.04 to 0.3mg/kg) (Endo and Tamiya 1991).

2.2. Pharmacological properties of ASIC-targeting toxins

The four ASIC-targeting peptides show different, although sometimes overlapping, pharmacological profiles on ASIC channels (Table 1).

Nanomolar concentrations of PcTx1 applied at physiological pH 7.4 potently inhibit rodent homomeric ASIC1a channels (IC_{50} ~ 1 nM) (Escoubas et al. 2000). Recently, PcTx1 has also been showed to inhibit heteromeric rat ASIC1a+ASIC2b channels with similar affinity to rat ASIC1a channels (IC_{50} ~ 3 nM) (Sherwood et al. 2011). The human ASIC1a channel (*i.e.*, the human ortholog of rat ASIC1a) is inhibited by the PcTx1-containing venom applied at pH 7.2 (Sherwood and Askwith 2008), and by PcTx1 at pH 6.2 (IC_{50} ~13 nM) (Qadri et al. 2009). However, a high concentration of PcTx1 applied at pH 7.4 potentiates the activation of hASIC1a current by acidification (Hoagland et al. 2010). At high concentration, PcTx1 interacts with human and rodent ASIC1b channel by potentiating its activation by acidification (EC_{50} ~ 100 nM) (Hoagland et al. 2010) (Chen et al. 2006). Finally, PcTx1 also directly activates chicken ASIC1a channels (EC_{50} ~ 189 nM) (Samways et al. 2009). The peptide has no effect on the other homomeric or heteromeric ASIC channels, as well as on a variety of Kv, Nav and Cav channels (Escoubas et al. 2000). The specificity of the toxin for ASIC channels is supported by binding experiments with an iodinated form of the peptide, which show similar binding properties on rat brain membranes and heterologously expressed channels (Salinas et al. 2006).

Mambalgins, like PcTx1, inhibit homomeric rodent and human ASIC1a channels as well as other ASIC1a-containing and ASIC1b-containing channels with IC_{50} ranging from 11 to 252 nM (Table1). Mambalgin-1, -2 and -3 display the same pharmacological profile (Diochot et al., 2012 and S. Diochot and M. Dauvois, personal communication). Mambalgins inhibit all the ASIC channel combinations expressed in central neurons (*i.e.*, ASIC1a, ASIC1a+ASIC2a and ASIC1a+ASIC2b). They were accordingly found to drastically inhibit the ASIC currents present in spinal cord and hippocampal neurons, whereas PcTx1 was found to only inhibit about 30% of the amplitude of these currents (Diochot et al. 2012; Baron et al. 2008; Baron et al. 2002). In sensory neurons, mambalgins inhibit about 60% of ASIC currents and PcTx1 about 40%, which has been attributed to the inhibition by mambalgins of ASIC1b-containing channels (Diochot et al. 2012).

APETx2 inhibits homomeric rat and human ASIC3 channels (IC_{50} ~ 37-63 nM for rat and ~175 nM for human), and several heteromeric ASIC3-containing channels: heteromeric ASIC3+ASIC2b (IC_{50}~117 nM), ASIC3+ASIC1b (IC_{50}~ 0.9 µM) and ASIC3+ASIC1a (IC_{50} ~ 2 µM) (Jensen et al. 2009) (Anangi et al. 2010; Diochot et al. 2004; Chagot et al. 2005). Upon acidification, ASIC3 channels generate a classical transient and rapidly inactivating current and an additional slowly activating, sustained current that does not inactivate as long as the pH remains acidic (Salinas et al. 2009). APETx2 rapidly and reversibly inhibits the transient peak current and the sustained component evoked at pH 7.0 (Deval et al. 2011), a window current resulting from the overlap between inactivation and activation of the peak current (Deval et al. 2008; Yagi et al. 2006), but the toxin does not affect the sustained component evoked at pH 5.0. In sensory neurons, APETx2 inhibits the ASIC currents that are insensitive to mambalgins (*i.e.*, ASIC3-containing currents not flowing through ASIC1a- or ASIC1b-containing channels) (Diochot et al. 2012; Diochot et al. 1998). APETx2 has been recently shown to also target to some extent the Nav 1.8 voltage-dependent Na^+ channel (IC_{50} between 55 nM and 19 µM, depending of the study) as well as $Na_V1.8$-related currents in rat DRG neurons (IC_{50} ~ 2.6 µM) (Peigneur et al. 2012) (Blanchard et al. 2012). APETx2 also inhibits Nav1.2 currents (IC_{50} ~ 114nM) and slightly reduces the Nav1.6 currents (17% inhibition by 1µM toxin) (Peigneur et al. 2012).

MitTx does not inhibit, but potently activates several homo- and heteromeric ASIC channels (Bohlen et al. 2011; Bohlen and Julius 2012). The most robust effects are seen on homomeric rodent ASIC1a and ASIC1b channels (EC_{50}~9.4 and 23 nM, respectively), with a much lower effect on ASIC3 (EC_{50}~830 nM). MitTx has only weak effects on ASIC2a at physiological pH 7.4, but strongly potentiates the acid-evoked current. An activation of heteromeric ASIC1a+ASIC2a channels, even if weaker than the one of homomeric ASIC1a, is also reported. The effect of MitTx on native ASIC currents in sensory trigeminal ganglion neurons seems to mainly depend on ASIC1a-containing channels because this effect disappears in neurons from ASIC1a-knockout mice (Bohlen et al. 2011).

3. Mechanisms of action and molecular basis of the interaction of ASIC toxins with their target channels

Based on the crystal structure of chicken ASIC1 (the ortholog of rat ASIC1a), a model has been proposed for an ASIC subunit (Fig. 3A) resembling an upright forearm and clenched arm holding a ball (Jasti et al. 2007). The two transmembrane domains (TM1 and TM2) form

the forearm, the junction with the extracellular domain forms the wrist, and the extracellular domain form the hand divided in palm, knuckle, finger, thumb and β-ball domains (Fig 3B). Analysis of the interaction between PcTx1 and ASIC1a channel has given a lot of information on the structure and function of these channels, including important information on channel open states through recent crystallisation of a chicken ASIC1a-PcTx1 complex (Baconguis and Gouaux 2012) (Fig. 3C).

3.1. State dependent binding of PcTx1 regulates the gating of ASIC1a and ASIC1b channels

PcTx1 is a gating modifier that increases the apparent affinity of rat ASIC1a channels for H^+ (Chen et al. 2005; Salinas et al. 2006). PcTx1 induces a shift of the pH-dependent inactivation curve of ASIC1a current towards less acidic pH, which transfers almost all channels into an inactivated state at pH 7.4 (Fig. 4A, B). This effect is associated with a small shift of the activation curve responsible for a minor stimulatory effect of PcTx1 observed with a preconditioning pH of 8.0, *i.e.*, in the absence of pH-dependent inactivation ((Chen et al. 2005)[Ba3] and Fig. 4A, B). PcTx1 has a similar effect on the human ASIC1a channel (Sherwood and Askwith 2008), but the inhibition is only observed at pH 7.2 with crude *Psalmopoeus cambridgei* venom. The different behaviour between rat and human ASIC1a is supported by five divergent residues in the thumb domain, which render the pH-dependent inactivation of human ASIC1a channel less sensitive to pH and thus prevent the potent inhibitory effect of PcTx1 applied at preconditioning pH 7.4, revealing instead a current stimulation at high concentration (Hoagland et al. 2010). PcTx1 promotes opening of rat ASIC1b channels at physiological pH 7.4 (EC_{50}~100 nM) through a shift of its activation curve towards less acidic pH (Fig. 4C, D), whereas the inactivation curve is almost not affected. The difference of PcTx1 affinity between the inactivated state of ASIC1a and ASIC1b channels depends on the adjacent upper part of the palm domain (see Fig. 3B) (Salinas et al. 2006; Chen et al. 2006). PcTx1 is also able to constitutively activate chicken ASIC1 channel (the ortholog of rat ASIC1a) at resting pH 7.4 (Samways et al. 2009) and to potentiate the H^+-activated current at pH 5.0 (M. Salinas, personal communication, Fig. 4E), probably by stabilizing the open state of the channel (Baconguis and Gouaux 2012) (Fig. 4F). The activating effect at pH 7.4 and the potentiating effect at pH 5.0 are consistent with the two open states stabilized by PcTx1 at high and low pH described by (Baconguis and Gouaux 2012)[Ba4]. PcTx1 thus appear to have complex state-dependent effects on ASIC1a and

ASIC1b channels that depends on the animal species and the corresponding pH-dependent properties of the channel, and on the pH at which the toxin is applied. This leads to three different global effects of PcTx1: inhibition of the H^+-gated ASIC current, stimulation of the H^+-gated ASIC current, or activation of the ASIC current at physiological pH 7.4 (Table 1).

PcTx1 has a group of four basic residues (Lys25, Arg26, Arg27, Arg28), which forms a positive surface protruding from the rest of the molecule (Escoubas et al. 2003) with three aromatic residues (Trp7, Trp24, and Phe30) in the vicinity. This "basic-aromatic dyad", equivalent to those described in scorpion or sea anemone toxins acting on voltage-gated ion channels (Escoubas et al. 2003; Dauplais et al. 1997), has been proposed to be the functional surface of PcTx1 (Escoubas et al. 2003; Chagot et al. 2005). Moreover, the electrostatic anisotropy of PcTx1 generates a dipole that emerges through the basic-aromatic dyad and could play an orientating force within the electrostatic field of ASIC1a channel. The role of PcTx1 hydrophobic residues Trp7 and Trp24 and basic residues Arg26, Arg27 and Arg28 have been recently confirmed and refined by the co-crystallization of PcTx1 bound to the chicken ASIC1 channel (Baconguis and Gouaux 2012). The hydrophobic patch on PcTx1 seals the basic cluster enhancing the electrostatic interactions with acidic residues of the channel.

We had proposed from data obtained by combining the ^{125}I-PcTx1 toxin binding and electrophysiological studies of ASIC chimeras, that the thumb domain and the upper part of the palm domain from adjacent subunit are involved in the binding site of PcTx1 on homomeric rat ASIC1a channel (Salinas et al. 2006) (Fig 3B, C). Computer−aided molecular docking analyses (Pietra 2011; Saez et al. 2011; Qadri et al. 2009), and more especially the recent determination of the crystal structure of chicken ASIC1 bound to PcTx1 (Dawson et al. 2012; Baconguis and Gouaux 2012) are supporting these hypothesis and bring up new information. Three PcTx1 molecules actually bind the ASIC1a channel at the interfaces between subunits, within the acidic pocket of the channel (Fig. 3B, C), a region (also called "pH sensor") that is thought to play a key role in H^+-dependent gating (Baconguis and Gouaux 2012; Dawson et al. 2012; Pietra 2011; Saez et al. 2011; Qadri et al. 2009; Salinas et al. 2006; Sherwood et al. 2009).

In the upper part of the thumb domain of rat ASIC1a, the key role of Asp349 and Phe350 (Asp350 and Phe351 in chicken ASIC1 or Asp351 and Phe352 in human ASIC1a) in the interaction between PcTx1 and the channel has been shown by mutagenesis (Salinas et al. 2006; Sherwood et al. 2009) and confirmed from the crystal structure (Baconguis and Gouaux 2012). The hydrophobic patch of PcTx1 (Trp7 and Trp24) interacts with the thumb domain of

the channel, whereas the basic cluster (Arg26, Arg27 and Arg28) enters into the acidic pocket to form strong H-bonds (Baconguis and Gouaux 2012).

Proton binding into the acidic pocket of the ASIC1a channel causes the disruption of carboxyl-carboxylate pairs and the release of coordinated Ca^{2+} (Jasti et al. 2007; Gründer and Xuanmao 2010). Both the Ca^{2+}-dependent binding of PcTx1 (Chen et al. 2005) and the highly pH-dependent binding of ^{125}I-PcTx1 (Salinas et al. 2006) support the fact that arginine residues of PcTx1 mimic the binding of protons to the ASIC1a acidic pocket that leads to steady-state inactivation (Saez et al. 2011).

The crystal structure of the PcTx1-bound chicken ASIC1 channel (Baconguis and Gouaux 2012) also allowed a better understanding of H^+-gated inactivation of the ASIC1a channel. From the open state, the lower palm domain and the wrist domain slightly rotate, strengthening the thumb and the palm domains of adjacent subunits, which leads to the narrowing of the central vestibule and induces a rearrangement of the transmembrane domains responsible for the inactivation. Finger and thumb domains, which flank the palm domain and make major contributions to the acidic pocket, bind PcTx1 and presumably also H^+, thereby modulating movements of the lower palm domain (Baconguis and Gouaux 2012).

3.2. Interaction between ASIC3 and APETx2

The interaction between APETx2 and ASIC3 has not been extensively studied yet. It is however possible to speculate, based on the analysis of electrostatic anisotropy on its surface, that a particular patch of residues in APETx2 could be involved in the interaction with ASIC channels. The presence of a "basic aromatic dyad" (Arg17, Arg31, Phe15, Tyr16 and Tyr32) and a basic –hydroxyl cluster (Ser9, Lys10), which define a dipole moment emerging through the patch of basic residues (Chagot et al. 2005), may indeed suggest that APETx2 could also bind to the acidic pocket to produce current inhibition *via* a gating modifier mechanism involving an apparent shift for H^+ affinity. The importance of the Phe15 residue in APETx2 for its inhibition of ASIC3 has been recently demonstrated (Anangi et al. 2012) by using an *Escherichia coli* periplasmic expression system for the production of the peptide. This interesting system will certainly facilitate analysis of the structure-function of APETx2 by facilitating the production of mutant toxins. Despite the fact that APETx2 and PcTx1 display no sequence homology and no common structural element, it is therefore possible that both toxins show similarities in their binding mechanism on their respective targets, *i.e.*, ASIC3- and ASIC1a-containing channels.

3.3. MitTx potentiates the activation of ASIC channels

The heterodimeric MitTx complex is able to activate ASIC1a, ASIC1b and heteromeric ASIC1a+ASIC2a channels at resting pH 7.4, and to potentiate the acid-evoked ASIC2a current by shifting its activation curve towards less acidic pH (Bohlen et al. 2011). The 3D-structure of β-bungarotoxin is very similar to MitTx (Kondo et al. 1978), and the co-crystallization of β-bungarotoxin with its target, the nicotinic-acetylcholine-receptor, revealed that it deeply binds into the acetylcholine-binding domains at subunit interfaces *via* extensive hydrophobic interactions, complemented by hydrogen bonding and electrostatic interactions. By homology, it can be proposed that MitTx could also bind to ASIC channels *via* its ligand-binding domains, *i.e.* the pH sensor. If this is true, one may expect some overlap between the binding sites of MitTx and PcTx1 on ASIC1a channel, which is consistent with the fact that their effect were actually not additive (Bohlen et al. 2011).

3.4. Mambalgins are gating modifier toxins that trap ASIC channels in the closed state

Mambalgin-1 inhibits ASIC1a channel by inducing a strong shift of its pH dependent activation toward more acidic pH, thus trapping the channel in its closed state (Fig. 4G, H) (Diochot et al. 2012). Mambalgin-1 also induces a small shift of the steady-state inactivation toward more alkaline pH, thus stabilizing the channel in the inactivated state. The binding of mambalgin-1 thus appears to be state-dependent with a preference for the closed state and marginally for the inactivated state. A 3D-model of mambalgin-1 reveals a strong positive electrostatic potential which may contribute, like for PcTx1 and APETx2, to favor the binding of the peptide to negatively charged regions of ASIC channels (Diochot et al. 2012). Mambalgins are able to inhibit homomeric ASIC1a but also ASIC1b channels suggesting difference in the inhibitory mechanism compared to PcTx1, which potentiates rather than inhibits ASIC1b channel. The apparent lack of competition between PcTx1 and mambalgins in inhibiting the ASIC1a current (Diochot et al. 2012) may suggest that each toxin does not bind to the same site and/or state of the channel.

4. *In vivo* effects of ASIC-targeting toxins

In vivo exploration of the physiological and pathophysiological roles of ASIC channels has been largely based on the phenotypic analysis of knockout mice. However, available ASIC-knockout mice are constitutive knockout, raising the possibility of the existence of compensatory mechanisms (related or not to ASICs), and conditional knockout are not available yet. In this context, pharmacological approaches in wild-type animals are of high potential and well complement the knockout approach. Besides the nonspecific and/or subtype nonspecific small molecule inhibitors of ASIC channels (like amiloride), specific ASIC-targeting peptides isolated from animal venoms are certainly among the most interesting tools for the pharmacological exploration of the role of these channels *in vivo* (Fig. 5).

4.1. Central injection of PcTx1 and mambalgins reveals the role in pain of different ASIC1a-containing channels expressed in neurons of the central nervous system.

ASIC1a and ASIC2a/2b are largely expressed in central neurons including spinal neurons that receive and modulate primary afferent inputs and neurons in brain areas associated with pain. Analysis of the functional properties of ASIC currents in cultured neurons combined with the use of ASIC-specific peptides has shown that ASIC currents in central neurons generally flow through a mixture of ASIC1a-containing channels, either homomeric ASIC1a channels or heteromeric ASIC1a+ASIC2a or ASIC1a+ASIC2b channels (Baron et al. 2002; Baron et al. 2008; Sherwood et al. 2011). In spinal cord, their expression is up-regulated during peripheral inflammation and they have been proposed to play a role in the processing of noxious stimuli as well as in central sensitization associated with hyperalgesia and allodynia in persistent pain (Duan et al. 2007; Wu et al. 2004). Intrathecal injections of ASIC-inhibitory toxins in the lumbar spinal cord of mice and rats clearly demonstrate a role for central ASIC1a-containing channels in the pain pathway, which was not anticipated from the phenotypic analysis of knockout animals for ASIC1a that have no major pain phenotype (Wemmie et al. 2003; Wemmie et al. 2002).

In mice, intrathecal (*i.t.*) and intracerebroventricular (*i.c.v.*) injections of PcTx1 were shown to induce a potent analgesic effect in acute pain as well as inflammatory and neuropathic pain models (Mazzuca et al. 2007). Knockdown of ASIC1a after *i.t.* injection of antisense oligonucleotides has a similar analgesic effect, thus supporting together with the lack of effect of the toxin in ASIC1a knockout animals (A. Baron, personal communication) the specificity of the PcTx1 effects *in vivo*. The analgesic effects of PcTx1 were blocked by

naloxone (an opioid receptor antagonist) and were absent in mice deficient for the preproenkephalin gene, which demonstrates that blocking of ASIC1a homomeric channels and probably ASIC1a+ASIC2b channels (which are also targeted by PcTx1 (Sherwood et al. 2011)) at the spinal and/or supra-spinal level activates the endogenous opioid pathway and increases the level of Met-enkephalin in the cerebrospinal fluid (Mazzuca et al. 2007) which is associated with strong analgesic effects. Intrathecal injections of PcTx1 were also recently shown to prevent chronic abdominal pain in a rat model of irritable bowel syndrome (IBS) induced by butyrate (Matricon et al. 2011).

Central (*i.t.* and *i.c.v.)* injections in mice of mambalgins were shown to also induce potent analgesic effects in acute as well as inflammatory pain (Diochot et al. 2012). Contrary to the effects of PcTx1, the effects of mambalgins were shown to be resistant to naloxone. The effect is completely lost in ASIC1a knockout mice demonstrating the essential implication of ASIC1a-containing channels and the specificity of mambalgin *in vivo*. Knockdown of ASIC2a after *i.t.* injections of siRNAs has a similar analgesic effect and reduces subsequent effect of the toxin, supporting the participation of the ASIC2a subunit in the effect *via* heteromeric ASIC1a+ASIC2a channels and providing the first evidence of the involvement of ASIC2a in pain. Interestingly, the central analgesic effect of mambalgins can be as strong as morphine but seems to produce less unwanted side effects (Diochot et al. 2012).

Different ASIC channels are therefore involved in two different central pathways associated with pain transmission and/or modulation. Further studies are needed to identify the cellular basis and neuronal networks involved in such pathways, but central ASIC channels appear as promising therapeutic targets for novel analgesic drugs.

4.2. Peripheral injection of APETx2, MitTx and mambalgins reveals the role in pain and sensory perception of different ASIC channels expressed in sensory neurons

4.2.1. APETx2 provides pain relief through inhibition of ASIC3-containing channels

Peripheral injections of APETx2 combined with *in vivo* gene silencing through intrathecal injections of siRNAs have demonstrated the role of ASIC3 as a sensor of cutaneous acidic pain and post-operative pain as well as an integrator of molecular signals released during inflammation in rat, where it contributes to primary thermal hyperalgesia (Deval et al. 2008; Deval et al. 2011; Deval et al. 2010). Consistent with these data, local

peripheral application of APETx2 reduces mechanical hypersensitivity in a model of non-inflammatory muscular pain and in a model of cutaneous inflammatory pain (Karczewski et al. 2010). This is in good agreement with the increased expression of ASICs in rat DRG neurons induced by chronic inflammation of the hindpaw (Voilley et al. 2001; Mamet et al. 2002; Mamet et al. 2003). The increase in ASIC3 expression together with up-regulation of its activity by several components of the "inflammatory soup", such as bradykinin, 5-HT (Deval et al. 2004), hypertonicity (Deval et al. 2008), arachidonic acid (Deval et al. 2008; Allen and Attwell 2002; Smith et al. 2007), nitric oxide (Cadiou et al. 2007), ATP (Birdsong et al. 2010) and polyamines (Li et al. 2010) may be important for the sensitization of cutaneous nociceptors during inflammation. Interestingly and consistent with the data in rat, a decrease in pH in the skin of human volunteers has been associated with non-adapting pain (Steen et al. 1995) and this cutaneous acid-induced pain appears to be largely mediated by ASIC channels, especially at moderate pH >6.0, since it is blocked by amiloride (Ugawa et al. 2002; Jones et al. 2004; McMahon and Jones 2004).

ASICs are also expressed in sensory neurons that innervate muscle, joints and bone. ASIC3 is expressed in more than 50% of small muscle afferents in rat (Deval et al. 2011; Molliver et al. 2005) and in more than 30% of DRG neurons innervating the knee joint in mouse (Ikeuchi et al. 2009). ASIC expression in DRG is increased in models of muscle inflammation (Walder et al. 2010) and acute arthritis (Ikeuchi et al. 2009) in mice. The combination of APETx2, amiloride and A-317567 (a small molecule non-discriminative inhibitor of ASICs), together with knockout and knockdown mice has demonstrated an important role for ASIC3 in the generation of secondary mechanical hyperalgesia associated with central sensitization achieved in a mouse model of non-inflammatory muscular pain induced by repeated acid injections into the muscle (Sluka et al. 2003; Price et al. 2001) and in a mouse model of joint inflammation (Ikeuchi et al. 2008). ASIC3 has also been involved in the development of primary cutaneous mechanical hyperalgesia induced by muscle inflammation (Walder et al. 2011; Sluka et al. 2007). In a rat model of ostheoarthritis, continuous intra-articular injections of APETx2 reduced pain-related behaviour and secondary mechanical hyperalgesia, as well as the increase in ASIC3 expression in knee joint afferent sensory neurons (Izumi et al. 2012).

Blockade of ASICs by amiloride or APETx2 markedly attenuates the exercise pressor reflex generated by contracting skeletal muscle in cats (Hayes et al. 2007; McCord et al. 2009) and rats (Tsuchimochi et al. 2011). The role of ASIC channels in the local vascular control is supported by the expression of ASIC3 in muscle metaboreceptors, the sensory

nerves that innervate muscle arterioles and detect changes in muscle metabolism (Molliver et al. 2005).

4.2.2. APETx2 inhibits pressure-induced vasodilation (PIV) through blockade of ASIC3-containing channels in sensory neurons.

Some studies of ASIC3-knockout mice have reported subtle alterations in normal cutaneous mechanical sensitivity (Chen et al. 2002; Price et al. 2001; Price et al. 2000), while other studies were not able to show a significant contribution to mechanosensory function (Roza et al. 2004; Drew et al. 2004). By combining APETx2, and other pharmacological inhibitors of ASIC channels like amiloride and diclofenac (Voilley et al. 2001), with knockout mice, ASIC3 has been shown to be a neuronal sensor for the skin vasodilation response to direct pressure (a mechanism known as pressure induced vasodilation or PIV) in both humans and rodents, and for skin protection against pressure ulcers in mice (Fromy et al. 2012).

4.2.3. MitTx evokes pain through activation of ASIC1a-containing channels.

Injection of MitTx in the mice hindpaw produces a robust pain-related behaviour (licking response) that is decreased in ASIC1a-knockout mice but persists in ASIC3-knockout mice, thus suggesting the involvement of ASIC1a-containing channels in cutaneous pain (Bohlen et al. 2011). This is consistent with the effect of MitTx on ASIC current of cultured sensory trigeminal neurons, which seems to depend mainly on ASIC1a-containing channels because this effect disappears in neurons from ASIC1a knockout mice, but not from ASIC3 knockout mice. These data suggest a role for peripheral ASIC1a-containing channels in pain. On the other hand, subcutaneous injections of PcTx1 showed no effect in acute, inflammatory or post-operative pain models (Diochot et al. 2012; Mazzuca et al. 2007; Duan et al. 2007; Deval et al. 2008), which does not support a role for homomeric ASIC1a or heteromeric ASIC1a+ASIC2b channels, but does not exclude a role for the peripheral ASIC1a subunit in other heteromeric channels insensitive to PcTx1. It is also not clear at that point how pain produced by subcutaneous injection of ASIC activators like MitTx relates to pain detection triggered by a physiological stimulus, because ASIC channels inactivate over few seconds when activated by H^+ (*i.e.*, their physiological activator) while the strong MitTx-evoked responses are mainly non-inactivating and therefore carry far more current than would be produced even by a severe acidification.

4.2.4. Mambalgins provides pain relief through inhibition of ASIC1b-containing channels

Subcutaneous injections of mambalgin-1 in the mice hindpaw induces analgesia in acute heat pain and inflammatory heat hyperalgesia (Diochot et al. 2012). These effects are not involving ASIC1a, but ASIC1b-containing channels, as shown by the effect of ASIC1b silencing with siRNAs that produces a similar analgesia and reduces subsequent effect of mambalgin-1, demonstrating an *in vivo* role for ASIC1b in pain detection.

4.3. Central injection of PcTx1 induces neuroprotective effects though inhibition of ASIC1a-containing channels

In neurons of the central nervous system, lowering extracellular pH to the level commonly seen in ischemic brain activates ASIC1a currents, which results in increase in intracellular free Ca^{2+} concentration (Xiong et al. 2004; Yermolaieva et al. 2004; Herrera et al. 2008; Samways et al. 2009). Homomeric ASIC1a channels display some permeability to Ca^{2+} but the increase in intracellular Ca^{2+} seems to primarily occur through the secondary activation of voltage-gated Ca^{2+} channels and Ca^{2+} release from intracellular stores (Zha et al. 2006). Activation of ASIC1a-containing channels during metabolic acidosis accompanying stroke has been proposed to contribute to neuronal death associated with glutamate-independent mechanisms of Ca^{2+} entry during ischemia (acidotoxicity) (Gao et al. 2005; Xiong et al. 2004).

PcTx1 shows neuroprotective effects on mouse cultured hippocampal CA1 neurons submitted to the OGD (oxygen and glucose deprivation) model of ischemia (Gao et al. 2005) as well as on spinal motoneurons submitted to extracellular acidosis (Behan et al. 2013). These effects of PcTx1 could involve inhibition of homomeric ASIC1a channels or heteromeric ASIC1a+ASIC2b channels, as recently proposed (Sherwood et al. 2011). The crude PcTx1-containing *Psalmopoeus cambridgei* venom also shows a neuroprotective effect on cultured mice cortical neurons submitted to acid injury (Xiong et al. 2004) as well as in an *in vitro* model of ischemia (OGD) on cultured hippocampal neurons (Gao et al. 2005). It should be noted that in several *in vivo* studies, the crude *Psalmopoeus cambridgei* venom was used instead of pure PcTx1 toxin, which represents about 1% of the total protein content of the venom. It is not clear how other components of the venom can affect the data, but results that have been obtained with both pure toxin and the venom are generally similar. Neurons

from ASIC1a-knockout mice are also more resistant to acidotoxicity and ischemia (Xiong et al. 2004) supporting the involvement of ASIC1a-containing channels.

In rat and mouse models of cerebral focal ischemia, *i.c.v.* injection of *Psalmopoeus cambridgei* venom or PcTx1 30 minutes before (PcTx1) or as late as 5 hours after (venom) a severe (60 min) transient middle cerebral artery occlusion (MCAO) reduces the infarct volume by more than 50% and the protection persisted for at least 7 days (venom, no information for PcTx1) (Pignataro et al. 2007; Xiong et al. 2004). In the same MCAO model, ASIC1a-knockout mice also shows 25-60% smaller infarct than WT mice, depending on the study. In a recent work using a model of ischemia produced by asphyxia-induced cardiac arrest in pig, *i.c.v.* injection of *Psalmopoeus cambridgei* venom 20 min before hypoxia partially protects the neurons in putamen (Yang et al. 2011). Additive neuroprotection was obtained after pretreatment with an NMDA receptor antagonist (MK-801). *Psalmopoeus cambridgei* venom also shows some neuroprotective effects in the MPTP model of Pakinson's disease in mouse (Arias et al. 2008), as well as in a model of multiple sclerosis associated with axonal degeneration (Friese et al. 2007). In the later model, ASIC1a-knockout mice also presents a reduced clinical deficit compared to WT mice. Very recently, the first translational study on neuroprotection targeting ASIC1a has shown that a treatment with amiloride exerts neuroprotective effects in patients suffering from multiple sclerosis (Arun et al. 2013).[Ba5] Acid-induced axonal degeneration is also reduced by *Psalmopoeus cambridgei* venom in an *ex vivo* model of optic nerve and retinal explants (Friese et al. 2007). Interestingly, this neuroprotective effect is due to an action on neurons but also on oligodendrocytes that are in charge of the myelinisation of axons, which also express ASIC1a-containing channels (Vergo et al. 2011; Feldman et al. 2008). Using a model of traumatic neuronal spinal cord injury in rats, Hu et al. (Hu et al. 2011) showed that *i.t. Psalmopoeus cambridgei* venom reduces the lesion volume and increased the locomotor recovery 3 days after injury, which is associated with a 40% reduction of neuronal death in cultured neurons. These effects were reproduced by silencing ASIC1a with antisense oligonucleotides.

Retina is a functionally distinct region of the central nervous system that has been shown to express ASIC channels (Brockway et al. 2002) where they have been involved in normal retinal activity (Ettaiche et al. 2006; Ettaiche et al. 2009; Ettaiche et al. 2004). Intraocular (intravitreal) injection of PcTx1 and antisense oligonucleotides against ASIC1a in rats show that ASIC1a is a positive modulator of cone phototransduction and adaptation (Ettaiche et al. 2006). Toxins were not tested yet on *in vivo* pathological models of transient global ischemia, but PcTx1 reduces the *in vitro* ischemia-induced cell death of cultured rat

retinal ganglion cells (RGC) (Tan et al. 2011) that have been shown to express ASIC currents (Ettaiche et al. 2006). Ischemic RGC injury plays a role in a variety of retinal diseases such as diabetic retinopathy, hypertensive vascular disease and glaucoma.

ASIC1a-containing channels can therefore represent novel therapeutic targets for ischemic brain or retina injury (Xiong et al. 2007), and PcTx1 a new therapeutic candidate with neuroprotective properties (Wang and Xu 2011) in ischemia and stroke therapy as well as retinal diseases

4.4. Fear reduction, antidepressant and anxiolytic effects of central injection of PcTx1 through inhibition of ASIC1a-containing channels in the amygdala.

Expression of ASIC1a is particularly high in the amygdala, a brain region involved in fear, arousal and emotions (Wemmie et al. 2003). In this structure, ASIC1a acts as a pH sensor that contributes to the production of fear behaviour associated with inhalation of carbon dioxide and acidosis (Ziemann et al. 2009). ASIC1a actually contributes to fear responses to a variety of aversive stimuli (Maren 2009), probably in part through a contribution to synaptic plasticity underlying the acquisition of conditioned fear (Wemmie et al. 2002). In mice, *i.c.v.* injection of PcTx1-containing *Psalmopoeus cambridgei* venom reduces innate fear-related behaviour (Coryell et al. 2007). Genetically disrupting ASIC1a (ASIC1a knockout mice) also reduces innate fear, whereas the venom becomes ineffective, supporting the specificity towards ASIC1a of the venom effect on fear. Restoring ASIC1a expression in the basolateral amygdala of ASIC1a- knockout mice rescues contextual fear conditioning (Coryell et al. 2008). Conversely, overexpression of ASIC1a in transgenic mice increases fear conditioning (Wemmie et al. 2004).

After *i.c.v.* injection, PcTx1 was also found to exert antidepressant effects in the forced swim test (Coryell et al. 2009). Genetically disrupting ASIC1a also induces a similar effect, whereas PcTx1 is ineffective in these animals, confirming the specificity of the PcTx1 effect. *Psalmopoeus cambridgei* venom exerts the same antidepressant effect than PcTx1 (Coryell et al. 2009). Restoring ASIC1a in the amygdala with a viral vector eliminates the antidepressant-like phenotype in ASIC1a-knockout mice, suggesting that amygdala plays a key role in the anti-depressant-like effects associated with ASIC1a deletion or inhibition.

Anxiolytic-like effects of PcTx1 have also been described. In the stress-induced hyperthermia model, acute *i.c.v.* administration of *Psalmopoeus cambridgei* venom prevents

stress-induced elevations in mice core body temperature (Dwyer et al. 2009). A contribution in these anxiolytic-like effects of the GABAergic system in the amygdala has been proposed.

4.5. PcTx1 inhibits vasoconstriction in vascular smooth muscle cells through ASIC1a-containing channels

ASIC channels are expressed in vascular smooth muscle cells (VSMC) from arteries where they could play a role in mechanotransduction of the myogenic response (pressure-induced adjustement of vascular tone) and VSMC migration (Drummond et al. 2008). ASIC currents were recorded from cerebral artery smooth muscle cells freshly isolated from mice. These currents have been associated with PcTx1-inhibited ASIC1a-containing channels in 11% of the cells, but were enhanced by PcTx1 in 76% of the cells (Chung et al. 2010). This suggests that vascular smooth muscle cells expressed a significant level of ASIC1b channels, which are known to be enhanced by PcTx1 (Chen et al., 2006, see also #2.2.).

In pressurized rat small pulmonary arteries, PcTx1 was shown to reduce agonist-induced vasoconstriction and increase intracellular Ca^{2+} after a chronic hypoxia (Jernigan et al. 2012). In isolated rat small pulmonary arteries, PcTx1 and ASIC1 silencing with siRNA both reduces store-operated calcium entry in VSMCs, whereas others siRNAs against ASIC2 and ASIC3 are without effect (Jernigan et al. 2009). These results suggest that the inhibition of ASIC1a-containing channels rather than enhancement of ASIC1b currents may be involved in the effects of PcTx1 on agonist-induced vasoconstriction. However, results obtained with ASIC2-knockout mice also suggest that a normal ASIC2 expression could be required for a pressure-induced (myogenic) constriction of the mouse middle cerebral arteries, and ASIC2 may also be involved in establishing the basal myogenic tone (Gannon et al. 2008).

4.6. Improvement of glucose control by APETx2 through inhibition of ASIC3-containing channels in adipocytes

Expression of ASIC3 subunit and recording of ASIC3 current in adipose cells, as well as the lean phenotype of ASIC3-knockout mice related to a smaller size of adipocytes, were first reported by Huang et al. (Huang et al. 2008; Wu et al. 2012). Interestingly, ASIC3-knockout mice also shows enhanced insulin sensitivity and are protected against age-dependent glucose intolerance. Intraperitoneally injected APETx2 in aged wild-type mice produces the same effects, thus supporting a role for ASIC3 in age-dependent glucose

intolerance and insulin resistance in adipocytes, which is probably more related to its capacity to sense lactate than to sense low pH. Indeed, lactate can enhance the opening of ASIC3 at near physiological pH (Immke and McCleskey 2001) and elevated basal level of lactate is consistently found in diabetic subjects who also display marked insulin resistance (DiGirolamo et al. 1992).

4.7. Inhibition of acidosis-induced increase in breathing frequency through blockade of ASIC1a channels in hypothalamic neurons

Recent data obtained in rats support the involvement of ASIC channels in central chemoreception and breathing control. Injections of PcTx1 in the lateral hypothalamus blocked the increases in breathing frequency induced by extracellular acidosis, the activation of ASIC1a channels of orexin neurons, and the orexin action on the medulla respiratory centre (Song et al. 2012). ASIC-containing neurons also exist in the nucleus of the solitary tract that project to respiratory centres to increase breathing frequency in hypercapnic rats (Huda et al. 2012).

4.8. Reduction of inflammation-induced apoptosis of chondrocytes by PcTx1 and APETx2 through inhibition of ASIC1a- and ASIC3-containing channels, respectively.

ASIC3 has been shown to be expressed in joint afferent sensory neurons but also in chondrocytes and synoviocytes, where they may be important in inflammatory and degenerative joint disease (Ikeuchi et al. 2009; Kolker et al. 2010). ASIC3-knockout mice does not develop secondary hyperalgesia induced by carrageenan-induced arthritis (Ikeuchi et al. 2008). In a rat model of ostheoarthritis, the analgesic effects of APETx2 on ASIC3-containing channels expressed in sensory neurons were complemented by a reduction of cartilage damage caused by inflammation (Izumi et al. 2012). Similar results have been obtained with amiloride that protects articular chondrocytes from acid-induced apoptosis (Hu et al. 2012), leading to chondroprotection in arthritis rats (Yuan, Chen, Lu, Li, Li et al. 2010). PcTx1-containing *Psalmopoeus cambridgei* venom also significantly inhibits the increase in intracellular Ca^{2+} and the acid-induced death of cultured rat articular chondrocytes (Yuan, Chen, Lu, Li, Wu et al. 2010), suggesting that the inhibition of ASIC1a-containing channels could also protect chondrocytes.

4.9. Other putative in vivo effects of ASIC-targeting toxins

In an *in vivo* model of kainate-induced epilepsy, an *i.c.v.* injection of PcTx1 reduced both the seizure activity and the CA3 neuronal injury (Xiong et al. 2008)[Ba6], consistent with other studies showing an inhibition of seizures by amiloride in rats and mice (Ali et al. 2006) (Luszczki et al. 2009; N'Gouemo 2008). This raises the possibility of a contribution of ASIC channels, and ASIC1a-containing channels in particular, to the generation and/or maintenance of seizure. However, other results have shown that *i.c.v.* injection of *Psalmopoeus cambridgei* venom or deletion of ASIC1a increased seizure severity in mice, which has been associated with a protecting role of ASIC1a-containing channels in seizure termination by acidosis through an increase in the inhibitory tone of inhibitory interneurons (Ziemann et al. 2008), and which is consistent with the observation that breathing carbon dioxide makes brain tissue more acidic and stops seizures. Further studies are thus needed to understand the various contributions of ASIC channels to epilepsy, which may depend on the location of ASIC activation, the magnitude and duration of acidosis, and the presence of modulators of ASIC function.

ASIC currents were recorded from cultured neurons from rat inferior colliculus, partly flowing through PcTx1-sensitive channels. ASIC channels could thus play a role in information processing and pathological processes in the central auditory system (Zhang et al. 2008). Grade IV gliomas are the most common and most aggressive of all brain tumors, exhibiting high rates of proliferation and migration, often setting up secondary foci distant from the primary tumor. PcTx1 and ASIC1 silencing inhibit migration and proliferation of glioma cells (Rooj et al. 2012). PcTx1 (not specified in the study but probably as the *Psalmopoeus cambridgei* venom) has been recently shown to block cortical spreading depression (CSD), a phenomenon associated with migraine with aura in human, in a rat model of cortical needle prick-induced CSD (Holland et al. 2012). Interestingly, a role in migraine-related behaviour of peripheral ASIC3 channels expressed in dural afferents has also been proposed based on the effect of amiloride (Yan et al. 2011).

4.10. Inhibitory ASIC-targeting toxins display no toxicity.

ASIC-targeting inhibitory toxins (PcTx1, 0.46 µg *i.t.* or 23 µg / kg; mambalgins, 2.2 µg *i.t.* and *i.c.v.* or 110 µg / kg; APETx2, 1.8 µg *i.pl.*; 0.9 µg *i.v.*; 2.7 µg *i.t.* or 135 µg / kg)

never produce excitotoxicity, spasms, convulsions, motor paralysis, nor ataxia upon *in vivo* injections in mice unlike other spider, snake or sea anemone toxins like δ-atracotoxins (3.2 µg *s.c.* or 160 µg / kg) dendrotoxins (8 µg / kg intracisternal) , or ASII (2.5 µg / kg intracisternal) previously characterized as Nav activators or Kv1 inhibitors, and responsible for high neurotoxicity (Schweitz 1984) (Schweitz et al. 1990; Mylecharane et al. 1989). PcTx1 and APETx2 are also not toxic in insects (intra-abdominal injection in the cricket *Acheta domestica* of 470 and 450 ng / 100 mg of insect body weight, respectively), whereas the scorpion toxin AaHIT (80 ng / 100 mg of insect body weight) provokes an intense but reversible paralysis in more than 50% of the animals (S. Diochot and M. Dauvois, personal communication). APETx2 is also not toxic or lethal after *in vivo* injections in crustaceans (intramuscular injection in the shore crab *Carcinus maenas*) (Sanchez-Rodriguez 1997), unlike other sea anemone Na_V toxins previously described (ASII, AxI, (Beress and Beress 1975; Schweitz 1984)).

None of the ASIC inhibitory peptides is therefore toxic for the natural preys of the venomous animal producing it (PcTx1 and cricket, APETx2 and crab, mambalgins and mouse). If the role of pain-producing toxins like MitTx could be to discourage threatening predators by triggering a disorienting and memorable sensory experience, one can wonder what could be the role in venom of ASIC-targeting analgesic toxins like PcTx1, APETx2 and mambalgins. One possibility could be that these peptides participate to hunting strategies, for instance to avoid alarming the bitten preys that could run away or fight back. It is interesting however to mention that PcTx1 does not block but activates chicken ASIC1a. It is not known if PcTx1 can cause pain in birds, but as previously suggested (Gründer and Chen 2010), the presence of PcTx1 could also be a defense strategy employed by the tarantula against potential predators. It would be interesting to also check if ASIC toxins cannot be present in the host tissues (*i.e.*, outside the venom gland) to fulfill particular physiological functions (*e.g*, as endogenous analgesics), based on recent work suggesting protein recruitment from the venom into non-toxic host physiological functions (Casewell et al. 2012).

5. Conclusion

There are now several venom peptides targeting ASIC channels with high levels of specificity, which can discriminate the many subtypes of ASIC1 and ASIC3 containing channels. These peptides can occasionally share some common targets (*e.g.*, mambalgins and

PcTx1) despite their completely different sequence and structure. Since the discovery of the first ASIC-targeting toxin (PcTx1) in 2000, these peptides have been very important to better understand the structure-function relationships of ASIC channels, the subtypes involved in native currents, and their implication in processes like nociception, mechanosensitivity, chemosensitivity, synaptic plasticity or neurodegeneration associated with a variety of situations ranging from pain perception and modulation to neurological diseases including post-traumatic stress, anxiety, depression, stroke, epilepsy, neuroinflammatory disorders and neurodegenerative diseases, as well as other emerging physiological situations in non-neuronal cells. ASIC channels generally need to be blocked to have therapeutic benefit, but protective effects have also been described like for instance in epilepsy. Whether ASIC channels are protective or deleterious may probably depend on several parameters including where they are activated, to what extent and duration, and to the presence of modulators.

ASIC channels appear therefore as important targets for drug development in a variety of neurological and psychiatric diseases, and probably also in other pathophysiological conditions outside the nervous system. ASIC-targeting peptides isolated from animal venoms that specifically block this class of channels are therefore not only instrumental as pharmacological tools to explore their function but also represent molecules of great potential therapeutic value. ASIC inhibitory toxins are also good examples of the high potential of non-toxic components of animal venoms (Utkin and Osipov 2007; Nirthanan et al. 2003).

Conflict of Interest statement

The authors declare no conflict of interest.

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LEGENDS

Figure 1. Structure of ASIC targeting toxins

A, Sequence of the ASIC-targeting toxins that block (PcTx1, APETx2, mambalgins) or activate (MitTx) ASIC channels. MitTx is constituted by the non-covalent association of MitTxα and MitTxβ. A sequence alignement is shown for mambalgins (Mamb-1, Mamb-2 and Mamb-3). Residues in Mamb-2 and -3 that are different from Mamb-1 are indicated by black boxes, and disulfide bonds are indicated by red lines. **B**, Three dimensional structure of PcTx1 (PDB code 2KNI (Saez et al. 2011)), APETx2 (PDB code 1WXN (Chagot et al. 2005)) and structural model of mambalgin-1 (Diochot et al. 2012) and MitTx (Bohlen et al. 2011). All toxins are shown at the same scale (disulphide bridges in orange).

Figure 2. Mambalgins define a new structural and functional class among snake three-finger toxins. Phylogenetic tree built from blast results using the mambalgin-1 protein sequence. The four toxins showing the highest levels of sequence identity with mambalgin-1 (CM-2a, CM-3, CM-1b, OH-26) only display 45-57% identity. For clarity, only the most representative toxins are shown for each sub-family, and some branches (associated with black dots) are not expanded. Color boxes represent toxins sharing a common functional and/or molecular mode of action indicated in the legend. Mambalgins are the only three-finger toxins to target ASIC channels and show no neurotoxic effect.

Table 1. Effects on ASIC currents of Mamb-1, PcTx1, APETx2 and MitTx.

Data obtained by application of the toxins at physiological pH 7.4 (unless specified) before the pH stimulation on rat (r), chicken (c) and human (h) homomeric and heteromeric ASIC heterologously expressed in *Xenopus* oocytes or mammalian cells. Inhibition (Inh.) or potentiation (Pot.) of the ASIC peak current activated by a drop in pH, or activation (Act.) in the absence of acid stimulation is shown in red, purple and blue, respectively. The IC_{50} or EC_{50} values are indicated when available, if not, the maximal dose is written; No: no effect (the highest dose tested is indicated). References: a, (Diochot et al. 2012); b, (Escoubas et al. 2000); c, (Chen et al. 2005); d, (Chen et al. 2006); e, (Diochot et al. 2004); f, (Jensen et al. 2009); g, (Anangi et al. 2010); h, (Sherwood et al. 2011); i, (Bohlen et al. 2011); j, (Qadri et al. 2009); k, (Samways et al. 2009); l, (Baconguis and Gouaux 2012); m, (Sherwood and Askwith 2008); n, (Hoagland et al. 2010); o, (our unpublished data).

Figure 3. PcTx1 binds to the pH sensor of ASIC1a.

A, Domain organization of a single ASIC1 subunit, showing the two transmembrane domains (TM1 and TM2) flanking the large extracellular loop, with the same color code than in B. **B**, Schematic representation of the chicken ASIC1 structure according to the model of a hand holding a ball (adapted from Jasti et al., 2007), with two ASIC subunits out of three shown for clarity. Red arrows indicate the domains proposed to be involved in channel opening by coupling the extracellular domain with the transmembre domains TM1 and TM2. **C**, 3D-structure of the chicken ASIC1-PcTx1 complex at low pH (pH 5.5) (side view: Baconguis & Gouaux, 2012). Solvent-accessible surface representation of the channel, with subunits shown with different colours. The PcTx1 toxin is shown in yellow. Inset: enlargement of the region where PcTx1 binds into the acidic pocket (into a white frame).

Figure 4. State-dependent effects of PcTx1 and Mamb-1 on ASIC1a and ASIC1b channels.

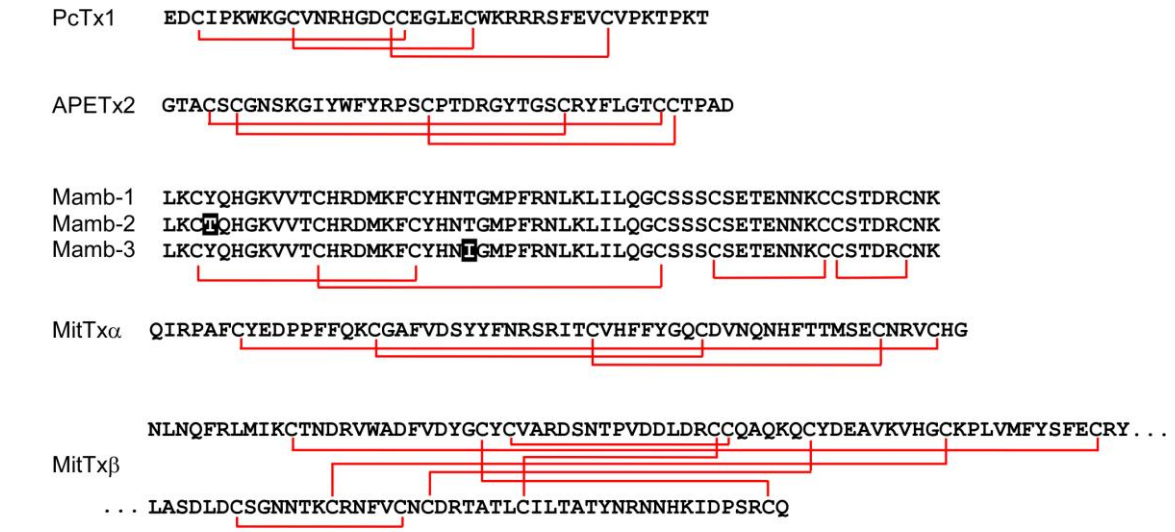
Schematic representation of the curves of pH-dependent activation (right) and inactivation (left) of ASIC currents, with or without toxins (protocols shown in inset). The shift of these curves by toxins leads to inhibition (red arrow) or stimulation (blue arrow) of the current. **A**, PcTx1 potently shifts the inactivation curve of rat homomeric ASIC1a current, with also a slight effect on the activation curve. **B**, Effect of PcTx1 on the different ASIC channels states, based on the Monod-Wyman-Changeux model for cooperativity in allostreric proteins (adapted from Chen et al., 2006). Open (O), closed (C) and desensitized (D) states are indicated with different hypothetical levels of protonation depending on the extracellular pH (indicated above) based on data from the literature (Chen et al. 2006, 2005; Diochot et al. 2012). A frame around the toxin name indicates the state of the channel that is the most stabilized by binding of the toxin. Stimulatory or inhibitory effects of PcTx1 on the current are represented by blue and red arrows, respectively (size of the arrow proportional to potency of the effect). PcTx1 binds most tightly to the desensitized and to a lesser extent the open state of rat ASIC1a, promoting either inhibition (pH 7.4+PcTx1 followed by pH 5.0 stimulation), or stimulation in a range of pH where negligible desensitization occurs (pH 8.0+PcTx1 followed by pH 6.7, or pH 7.4 followed by pH 7.1+PcTx1) (Chen et al. 2005). On human ASIC1a, PcTx1 exerts similar dual effects but for different pH values, promoting inhibition (pH 7.2+PcTx1 followed by pH 5.0 stimulation) or stimulation in the absence of inactivation (pH 7.4+PcTx1 followed by pH 6.7 stimulation) (Hoagland et al. 2010; Sherwood and Askwith 2008). **C,** PcTx1 shifts the activation curve of rat homomeric ASIC1b current, with

almost no effect on the inactivation curve. **D**, Based on the model already described in B, PcTx1 binds more tightly to the open state of rat ASIC1b, promoting opening (pH 7.5+PcTx1 followed by pH 6.0, or pH 7.5 / pH 6.6+PcTx1[Ba7] drops). However, inhibition is also possible for instance when PcTx1 is applied before a pulse at pH 5.0 from a conditioning pH 6.9 (Chen et al. 2006). **E,** PcTx1 (20 nM) is able to constitutively activate chicken ASIC1 channel at resting pH 7.4 (Samways et al. 2009) and to potentiate the H^+-activated current at pH 5.0. Red dashed line represents the current run-down. **F**, PcTx1 stabilizes the open state of chicken ASIC1, thus promoting activation (O1 state) at pH 7.4 without desensitization (absent at pH 7.4). The peak current at pH 5.0 is also potentiated (O2 state). The absence of inhibitory effects suggests that PcTx1 does not tightly bind to the desensitized state. The O1 and O2 states correspond to the open states stabilized by PcTx1 at high and low pH, respectively, which have been described by Banconguis et al., 2012. **G,** Mambalgin-1 induces a strong shift of the pH dependent activation of rat ASIC1a toward more acidic pH, with only a minor effect on the steady-state inactivation that is shifted toward more alkaline pH. **H**, Mamb-1 binds more tightly to the closed state and to a much lesser extent the desensitized state of rat ASIC1a, promoting closure of the channel at any pH (e.g., pH 7.4+Mamb-1 followed by pH 5.0).

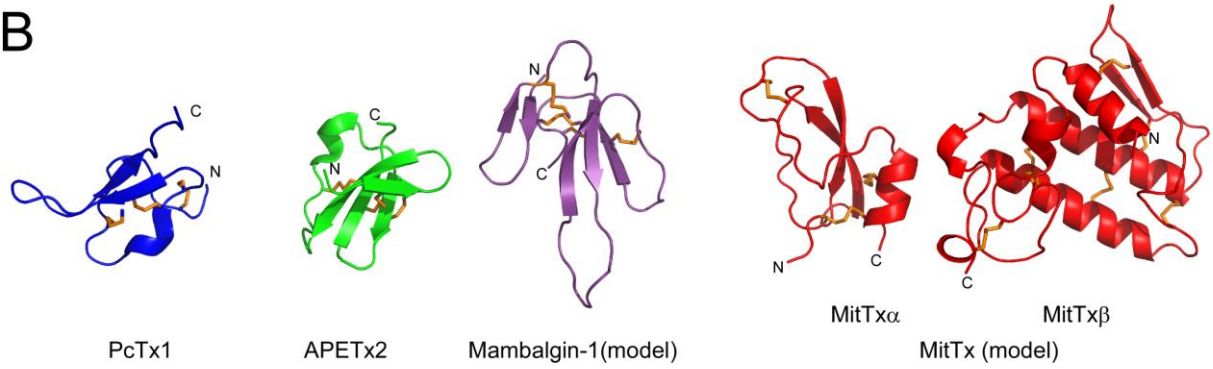
Figure 5[Ba8]**:** ***In vivo*** **effects of ASIC targeting toxins**

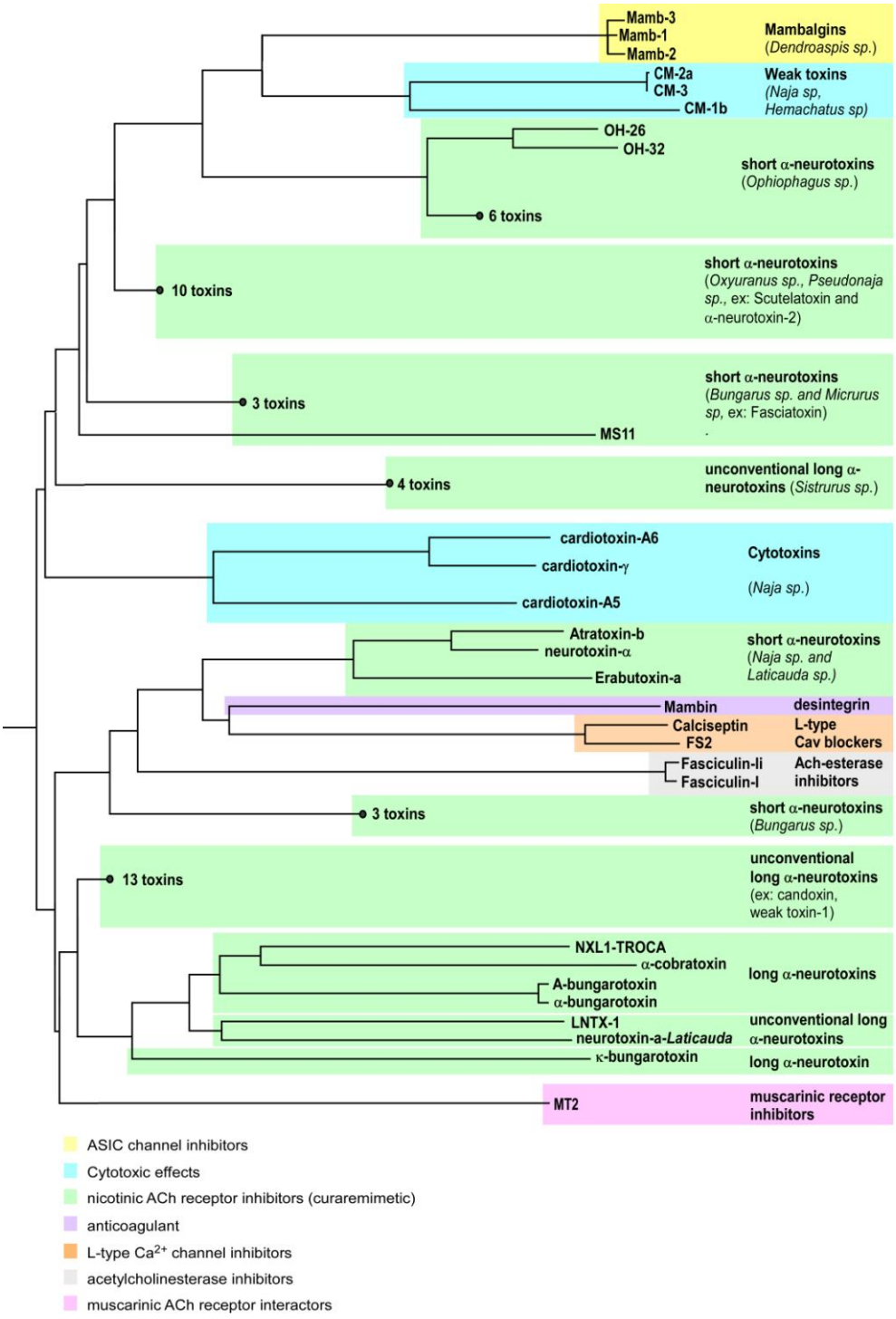
ASIC-cont., ASIC-containing; PIV, pressure-induced vasodilation, - and + represent inhibition and stimulation of ASIC channels, respectively.

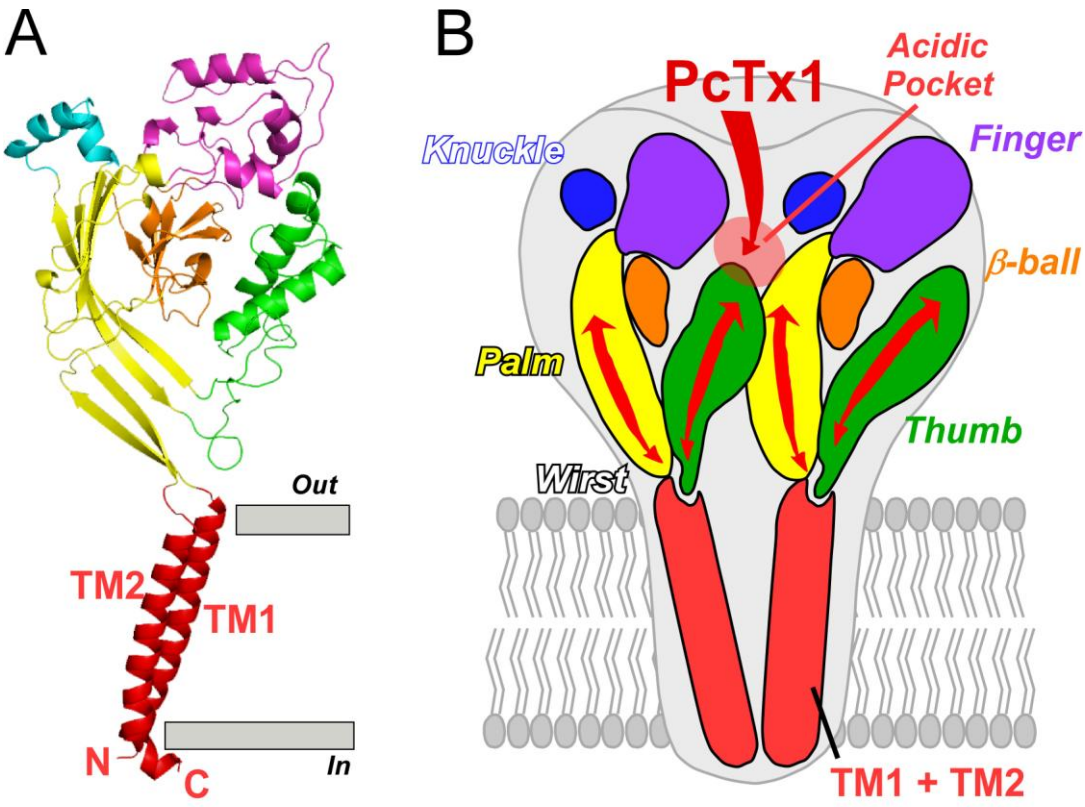
A

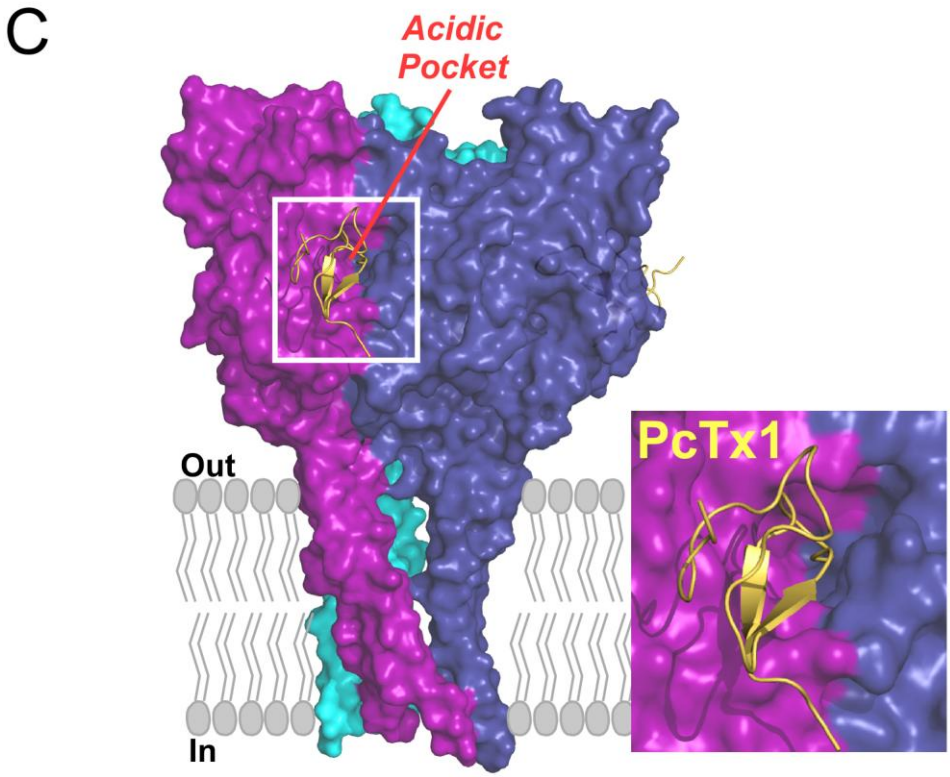

PcTx1 EDCIPKWKGCVNRHGDCCEGLECWKRRRSFEVCVPKTPKT
APETx2 GTACSCGNSKGIYWFYRPSCPTDRGYTGSCRYFLGTCCTPAD
Mamb-1 LKCYQHGKVVTCHRDMKFCYHNTGMPFRNLKLILQGCSSSCSETENNKCCSTDRCNK
Mamb-2 LKCIQHGKVVTCHRDMKFCYHNTGMPFRNLKLILQGCSSSCSETENNKCCSTDRCNK
Mamb-3 LKCYQHGKVVTCHRDMKFCYHNIGMPFRNLKLILQGCSSSCSETENNKCCSTDRCNK
MitTxα QIRPAFCYEDPPFFQKCGAFVDSYYFNRSRITCVHFFYGQCDVNQNHFTTMSECNRVCHG
NLNQFRLMIKCTNDRVWADFVDYGCYCVARDSNTPVDDLDRCCQAQKQCYDEAVKVHGCKPLVMFYSFECRY...
MitTxβ
...LASDLDCSGNNTKCRNFVCNCDRTATLCILTATYNRNNHKIDPSRCQ

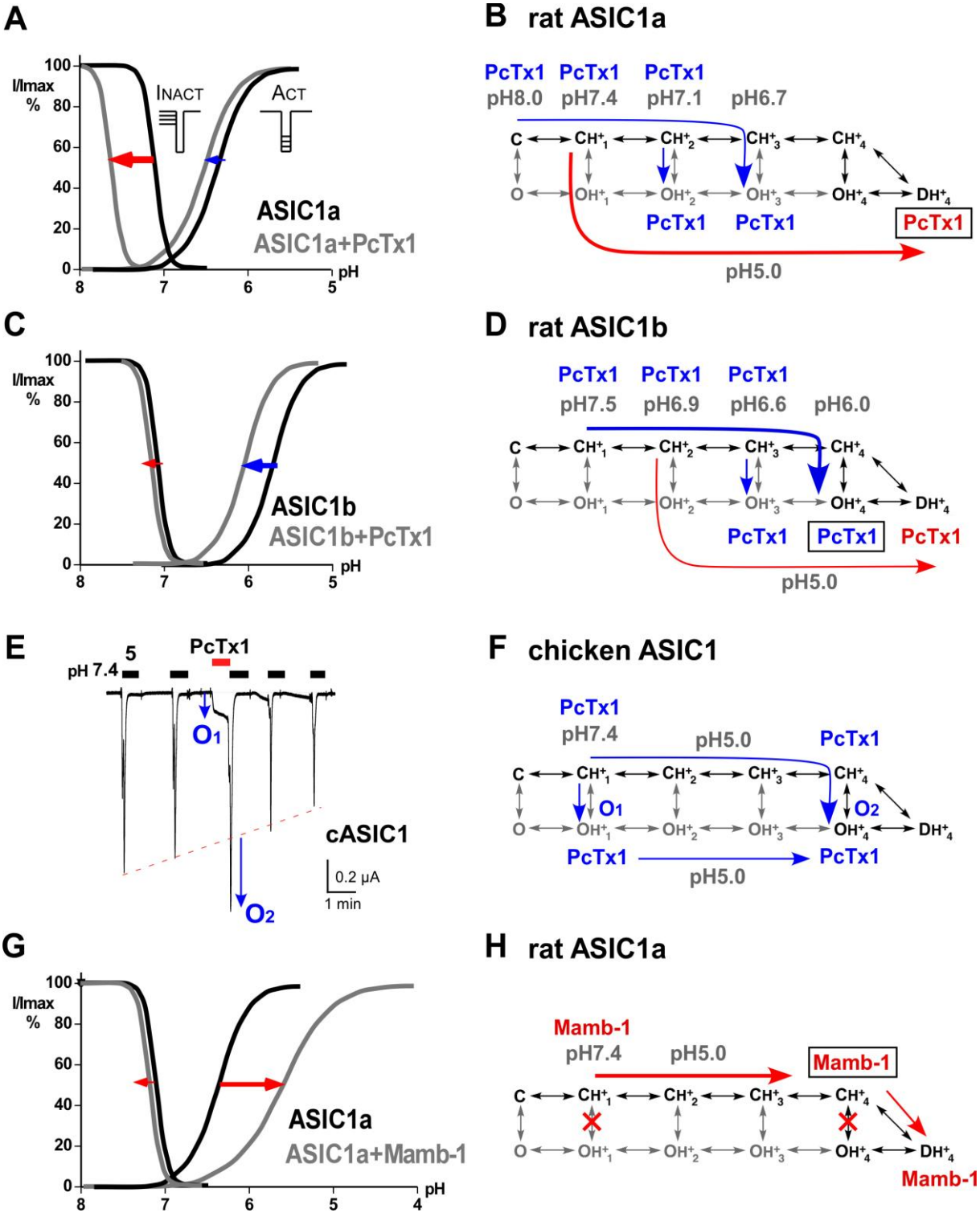
B


C
N
C
N
N
C
N
C
N
C
N
C
PcTx1
APETx2
Mambalgin-1(model)
MitTxα
MitTxβ
MitTx (model)

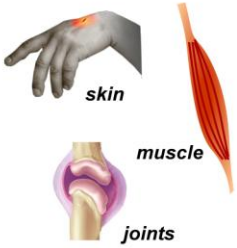

Mamb-3
Mamb-1
Mamb-2
Mambalgins
(Dendroaspis sp.)
CM-2a
CM-3
CM-1b
Weak toxins
(Naja sp,
Hemachatus sp)
OH-26
OH-32
6 toxins
short α-neurotoxins
(Ophiophagus sp.)
10 toxins
short α-neurotoxins
(Oxyuranus sp., Pseudonaja
sp., ex: Scutelatoxin and
α-neurotoxin-2)
3 toxins
MS11
short α-neurotoxins
(Bungarus sp. and Micrurus
sp, ex: Fasciatoxin)
4 toxins
unconventional long α-
neurotoxins (Sistrurus sp.)
cardiotoxin-A6
cardiotoxin-γ
cardiotoxin-A5
Cytotoxins
(Naja sp.)
Atratoxin-b
neurotoxin-α
Erabutoxin-a
short α-neurotoxins
(Naja sp. and
Laticauda sp.)
Mambin
desintegrin
Calciseptin
FS2
L-type
Cav blockers
Fasciculin-Ii
Fasciculin-I
Ach-esterase
inhibitors
3 toxins
short α-neurotoxins
(Bungarus sp.)
13 toxins
unconventional
long α-neurotoxins
(ex: candoxin,
weak toxin-1)
NXL1-TROCA
α-cobratoxin
A-bungarotoxin
α-bungarotoxin
long α-neurotoxins
LNTX-1
neurotoxin-a-Laticauda
unconventional long
α-neurotoxins
κ-bungarotoxin
long α-neurotoxin
MT2
muscarinic receptor
inhibitors
ASIC channel inhibitors
Cytotoxic effects
nicotinic ACh receptor inhibitors (curaremimetic)
anticoagulant
L-type Ca2+ channel inhibitors
acetylcholinesterase inhibitors
muscarinic ACh receptor interactors


A
Out
TM2
TM1
N
C
In
B
PcTx1
Acidic
Pocket
Knuckle
Finger
β-ball
Palm
Thumb
Wirst
TM1 + TM2


C
Acidic
Pocket
Out
In
PcTx1


A
100
I/Imax
%
80
60
40
20
0
8
7
6
5
pH
INACT
ACT
ASIC1a
ASIC1a+PcTx1
B rat ASIC1a
PcTx1 PcTx1 PcTx1
pH8.0 pH7.4 pH7.1 pH6.7
C
CH+1
CH+2
CH+3
CH+4
O
OH+1
OH+2
OH+3
OH+4
DH+4
PcTx1 PcTx1
PcTx1
pH5.0
C
100
I/Imax
%
80
60
40
20
0
8
7
6
5
pH
ASIC1b
ASIC1b+PcTx1
D rat ASIC1b
PcTx1 PcTx1 PcTx1
pH7.5 pH6.9 pH6.6 pH6.0
C
CH+1
CH+2
CH+3
CH+4
O
OH+1
OH+2
OH+3
OH+4
DH+4
PcTx1 PcTx1 PcTx1
pH5.0
E
5
PcTx1
pH 7.4
O1
O2
cASIC1
0.2 µA
1 min
F chicken ASIC1
PcTx1
pH7.4
pH5.0
PcTx1
C
CH+1
CH+2
CH+3
CH+4
O1
O2
O
OH+1
OH+2
OH+3
OH+4
DH+4
PcTx1
PcTx1
pH5.0
G
100
I/Imax
%
80
60
40
20
0
8
7
6
5
4
pH
ASIC1a
ASIC1a+Mamb-1
H rat ASIC1a
Mamb-1
pH7.4
pH5.0
Mamb-1
C
CH+1
CH+2
CH+3
CH+4
O
OH+1
OH+2
OH+3
OH+4
DH+4
Mamb-1

Peripheral nervous system

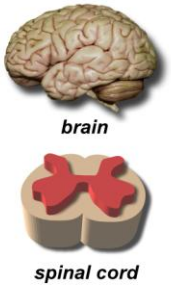


APETx2:
Analgesia
Inhibition of PIV in skin
Inhibition of exercise-pressure reflex in muscle
⊖ASIC3-cont. channels

Mamb-1:
Analgesia
⊖ASIC1b-cont. channels

MitTx:
Pain
⊕ASIC1a-cont. channels

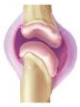
Central nervous system



PcTx1 :
Enkephalin-dependent analgesia
Neuroprotection against ischemia
Fear reduction
Anxiolytic effects
Control of breathing frequency
⊖ASIC1a-cont. channels

Mamb-1:
Opioid-independent analgesia
⊖ASIC1a+ASIC2a channels

Non neuronal tissues



PcTx1:
Chondroprotection against ischemia *(venom)*
⊖ASIC1a-cont channels

APETx2:
Chondroprotection against ischemia
Relief of ostheoarthritis pain
⊖ASIC3-cont channels

PcTx1
Inhibition of agonist-induced vasoconstriction
⊖ASIC1a-cont channels

APETx2
Protection against age-dependent glucose intolerance and insuline resistance
⊖ASIC3-cont channels

Retina

PcTx1
Impairement of cone phototransduction
⊖ASIC1a-cont channels

Channel	Mamb-1 IC_{50}	Mamb-2 IC_{50}	Mamb-3 IC_{50}	PcTx1 IC_{50}	APETx2 IC_{50}	MitTx EC_{50}
rASIC1a	**Inh.** 11-55 nM[a,o]	**Inh.** 55 nM[a]	**Inh.** 17-60 nM[o]	**Inh.** 0.7-3 nM[b,c]		**Act.** 9 nM[i]
rASIC1b	**Inh.** 44-192 nM[a,o]	**Inh.** 44-55 nM[a,o]	**Inh.** 44 nM[o]	**Pot.** 100 nM[d]		**Act.** 23 nM[i]
rASIC2a	**No** up to 2 µM[a]	**No** up to 3 µM[a]		**No** up to 100 nM[b]		**Pot.** at 75 nM[i]
rASIC3	**No** up to 2 µM[a]	**No** up to 3 µM[a]		**No** up to 100 nM[b]	**Inh.** 37-63 nM[e,f,g]	**Act.** 830 nM[i]
rASIC1a+ASIC2a	**Inh.** 246-252 nM[a,o]	**Inh.** 252 nM[o]	**Inh.** 252 nM[o]	**No** up to 10 nM[b]		**Act.** at 75nM[i]
rASIC1a+ASIC2b	**Inh.** 61 nM[a]			**Inh.** 3nM[h]		
rASIC1a+ASIC1b	**Inh.** 72 nM[a]					
rASIC1a+ASIC3	**No** up to 2µM[a]			**No** up to 10 nM[b]	**Inh.** 2 µM[e]	**No** up to 75 nM[i]
rASIC1b+ASIC3	**No** up to 2µM[a]	**No** up to 3µM[a]			**Inh.** 900 nM[e]	
rASIC2a+ASIC3					**No** up to 3µM[e]	**No** up to 75 nM[i]
rASIC2b+ASIC3					**Inh.** 117 nM[e]	
hASIC1a (ortholog of rASIC1a)	**Inh.** 127 nM[a]	**Inh.** 127 nM[a]		**Pot.** at 60nM[n] **Inh.** 13 nM (pH6.2)[j] venom (pH7.2)[m]		
hASIC1b (ortholog of rASIC1b)				**Pot.** at 60nM[n]		
hASIC2a	**No** up to 670 nM[a]	**No** up to 850 nM[a]		**No** up to 25 nM[j]		
hASIC1a+ASIC2a	**Inh.** 220 nM[a]			**No** up to 100 nM[j]		
hASIC3					**Inh.** 175 nM[e]	
cASIC1				**Act.** 189 nM[l] **Pot.** at 20nM[k,m]		

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