Role of voltage-gated calcium channels in ascending pain pathways

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ABSTRACT

Voltage gated calcium channels (VGCCs) are well established mediators of pain signals in primary afferent neurons. N-type calcium channels are localized to synaptic nerve terminals in laminae 1 and 2 of the dorsal horn where their opening results in the release of neurotransmitters such as glutamate and substance P. The contribution of N-type channels to the processing of pain signals is regulated by alternate splicing of the N-type channel gene, with unique N-type channel splice variants being expressed in small nociceptive neurons. In contrast, T-type VGCCs of the Cav3.2 subtype are likely localized to nerve endings where they regulate cellular excitability. Consequently, inhibition of N-type and Cav3.2 T-type VGCCs has the propensity to mediate analgesia. T-type channel activity is regulated by redox modulation, and can be inhibited by a novel class of small organic blockers. N-type VGCC activity can be potently inhibited by highly selective peptide toxins that are delivered intrathecally, and the search for small organic blockers with clinical efficacy is ongoing. Here, we provide a brief overview of recent advances in this area, as presented at the Spring Pain Research conference (Grand Cayman, 2008).

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1. Introduction

Pain is an important physiological response designed to protect us from injury (for review, see Altier and Zamponi, 2004; Smith, 2004). However, numerous pathophysiological conditions, such as diabetes, viral infections, nerve injury and inflammation can give rise to persistent, chronic pain that does not appear to serve a useful purpose and is often refractory to currently available treatment options (Porreca et al., 2002). Upon the occurrence of a painful peripheral stimulus, peripheral nociceptive neurons are activated and a train of action potentials is initiated and propagates along the axons of primary afferent nerve fibers to nerve terminals embedded in laminae 1 and 2 of the dorsal horn of the spinal cord (Krarup, 2003). These nerve terminals release pro-nociceptive neurotransmitters such as glutamate, substance P and CGRP, which then activate postsynaptic receptors on neurons of the spinothalamic tract. These nerve projections to the thalamus allow us to perceive pain (Krarup, 2003). There are also descending pathways from the cortex to the spinal cord that modulate pain responses (Porreca et al., 2002). The propagation and processing of pain signals are dependent on, and modulated by, a host of different ion channels and receptors, among these voltage gated calcium channels (VGCCs; Julius and Basbaum, 2001).

T-type VGCCs are expressed in cell bodies and nerve endings of afferent fibers where they partake in regulating neuronal excitability by contributing to the initiation of action potential trains (Todorovic and Jevtovic-Todorovic, 2006). Specifically, T-type VGCCs can lower the threshold for action potentials, promote bursting activity and synaptic excitation; all actions that favor the development of enhanced pain (Matthews and Dickenson, 2001; Sekizawa et al., 2000). Recently, studies have revealed that Cav3.2 VGCCs facilitate pain signals in peripheral nociceptors (Todorovic and Jevtovic-Todorovic, 2006). Also, in both rat models of diabetic neuropathy and the chronic constriction nerve injury model of neuropathic pain, T-type channel current density is remarkably increased (Jagodic et al., 2007, 2008). Conversely, gene knockout or antisense knockdown of the Cav3.2 isoform of T-type channels, or intrathecal injection of rats with T-type channel inhibitors, results in hyposensitivity to pain due to reduced excitability of the primary afferent fibers (Choi et al., 2007; Bourinet et al., 2005; Todorovic et al., 2002; Flatters and Bennett, 2004, Dogrul et al., 2003; Matthews and Dickenson, 2001; Shin et al., 2008).

On the other hand, high voltage-activated N-type VGCCs are highly expressed at presynaptic nerve terminals where they open in response to incoming action potentials and mediate calcium entry. These channels are highly localized to presynaptic terminals in laminae I and II of the dorsal horn. Action potentials carried along dorsal root ganglion cells (mainly C- and Aδ-afferents) trigger the opening of pre-synaptic N-type calcium channels which in turn initiate the release of nociceptive transmitters such as glutamate, substance P and CGRP onto spinal interneurons and projection neurons. Low voltage-activated T-type calcium channels are primarily localized more upstream in the pathway and are thought to be both involved in generating sensory potentials out near free nerve endings, as well as being present in DRG cell bodies where they likely contribute to the generation and frequency of action potentials. Modified from Hildebrand and Snutch, 2006; Schaible and Richter, 2004.

Fig. 1—N-type and T-type calcium channels in the primary afferent signaling pathway. High voltage-activated N-type channels are highly localized to presynaptic terminals in laminae I and II of the dorsal horn. Action potentials carried along dorsal root ganglion cells (mainly C- and Aδ-afferents) trigger the opening of pre-synaptic N-type calcium channels which in turn initiate the release of nociceptive transmitters such as glutamate, substance P and CGRP onto spinal interneurons and projection neurons. Low voltage-activated T-type calcium channels are primarily localized more upstream in the pathway and are thought to be both involved in generating sensory potentials out near free nerve endings, as well as being present in DRG cell bodies where they likely contribute to the generation and frequency of action potentials. Modified from Hildebrand and Snutch, 2006; Schaible and Richter, 2004.
entry into the synapse. This in turn triggers synaptic vesicle release and the activation of spinalhalmic neurons. A critical role of N-type VGCCs in the pain pathway is supported by data obtained from N-type channel null mice (which show an increased threshold for pain) and by the clinical efficacy of intrathecally delivered, selective N-type channel antagonists such as Prialt™ (Saegusa et al., 2001; Hatakeyama et al., 2001; Staats et al., 2004). Furthermore, N-type VGCCs are a key target for inhibition by opioid receptor pathways (Bourinet et al., 1996; Altier and Zamponi, 2004) and descending norepinephrine activation of adrenergic pathways (Pertovaara, 2006). Interestingly, other VGCC subtypes do not appear to play a major role in pain signaling in primary afferent fibers, perhaps with the exception of R-type channels (Saegusa et al., 2000; Matthews et al., 2007). As a result, N-type (Ca2.2) and Ca3.2 T-type VGCCs are considered prime targets for the development of novel analgesics (Fig. 1; Snutch, 2005; Hildebrand and Snutch, 2006).

2. N-type calcium channel splicing and pain

Although N-type VGCCs are encoded by a single α subunit gene (Ca2.2), structural and functional diversity can be generated though alternate mRNA splicing (Lipscombe, 2005; Gray et al., 2007). Numerous Ca2.2 splice variants have been identified and functionally characterized, but one variant has received particular attention due to its preferential expression in small nociceptive neurons (Bell et al., 2004). In this variant, exon 37b is replaced by exon 37a, leading to a 14 amino acid change in the Ca2.2 C-terminus region without changes in the total numbers of amino acids. The functional consequence of this splicing event included larger whole cell currents, as well as altered responses to the activation of certain G protein coupled receptors (Bell et al., 2004; Castiglioni et al., 2006; Raingo et al., 2007). Altier and colleagues (2007) investigated the physiological role of the exon 37a variant by using siRNA constructs that were designed to selectively knock down channels containing either exons 37a or 37b. Intrathecal injection of these siRNA constructs into rats revealed a critical role of exon 37a containing channels in thermal (assayed by a hot plate test) and mechanical nociception (assayed by von Frey stimulation of the hind paw). Moreover, the exon 37a containing channels were shown to be selectively involved in thermal hyperalgesia in inflammatory and neuropathic pain models, and in mechanical hyperalgesia. In contrast, both splice variants appeared to contribute to mechanical allodynia during neuropathy (Altier et al., 2007). Collectively, these data suggest that channels containing exon 37a are the predominant N-type channel species involved in sensing pain. Interestingly, during spinal nerve ligation, mRNA levels of exon 37a containing channels become downregulated by about 50% whereas those corresponding to exon 37b containing channels remain unaltered (Altier et al., 2007). This may suggest an intrinsic compensatory mechanism by which animals attempt to reduce the levels of the VGCC subtype that mediates pain in neuropathic states. The notion that exon37 containing channels appear to be critical for the transmission of pain signals at the spinal level, yet show only limited expression in other brain regions may provide for an opportunity to develop novel N-type channel blocking molecules that preferentially inhibit the exon37a splice isoform of the channels. This in turn may reduce the potential of side effects seen with global blockade of N-type channels via conotoxin derivatives.

3. Novel peptide toxin inhibitors of N-type channels

It is well established that intrathecal injection of N-type VGCC blocking peptides mediates analgesic behavioral responses in both rats and humans (Altier et al., 2007; Snutch, 2005; Staats et al., 2004). Indeed, Prialt™ is currently used clinically to treat cancer pain, however, its use has been associated with a number of side effects including unration behavior, hypotension, and memory loss (Staats et al., 2004). The reason for these side effects is unclear, but may include non-selective actions of Prialt™ on targets other than the N-type channels, or perhaps immune reactions to the injected peptides. Hence, the search for peptide inhibitors with a wider therapeutic window continues.

The ω-conotoxins produced by fish hunting cone snails were the first and remain amongst the most selective inhibitors of N-type VGCCs identified. Intrathecal administration of sub-nanomolar doses of the highly N-type selective ω-conotoxins MVIIA or CVID reduced pain behaviors in rats that last for up to 24 h but were accompanied by significant neurological side effects (Malmberg and Yaks, 1995; Smith et al., 2002). Based on this efficacy, ω-MVIIA (Prialt™) was developed by Neurex and later acquired by Elan. Prialt™ recently gained FDA approval for the treatment of otherwise unmanageable severe chronic pain after extended Phase III trials. ω-CVID (AM336) was also assessed in a small Phase IIa trial in severe cancer pain sufferers and again produced signs of efficacy. Unfortunately, both ω-conotoxins produced unwanted side effects at therapeutic doses, despite having up to 106 fold binding selectivity for N-type over P/Q-type VGCCs (Lewis et al., 2000). While off-target effects on P/Q-calcium channels cannot be discounted, on-target effects at inhibitory descending and interneuron synapses, as well as potential supraspinal effects, are likely to contribute to the dose-limiting side effects produced by these ω-conotoxins. The discovery of new ω-conotoxins and small molecules with selectivity profiles that produce fewer side effects may lead to the development of better N-type VGCC analgesics.

An alternative approach to the direct inhibition of N-type calcium channels is indirect inhibition via activation of Gi/Go coupled GPCRs. Whilst μ-opioid receptor agonists (e.g., morphine), α2-adrenergic agonists (e.g., clonidine) and non-selective small molecule norepinephrine transporter (NET) inhibitors (e.g. duloxetine) alleviate many types of pain, dose limiting side effects again also limit their usefulness (Martin and Eisenach, 2001). Since spinal noradrenaline release can reduce transmission by (i) activating inhibitory α2A-adrenoceptors on the central terminals of primary afferent nociceptors (presynaptic inhibition), (ii) direct α2-adrenergic action on pain-relay neurons (postsynaptic inhibition), and (iii) α1-adrenoceptor-mediated activation of inhibitory interneurons, spinaly administered selective and specific NET inhibitors might be expect to be useful analgesics. The Lewis
group isolated several $\chi$-conopeptides from the cone snail *Conus marmoreus* that proved to be highly selective peptide inhibitors of NET (Sharpe et al., 2001). Interestingly, the binding site for $\chi$-conopeptides on the NET partially overlaps the tricyclic antidepressant binding site but not the NE binding site (Paczkowski et al., 2007). Importantly, MrIA produced a potent anti-allodynic effect without significant side effects when administrated intrathecally in rat models of neuropathic pain (Nielsen et al., 2005) and no off-target effects on a range of tissues and targets at $< 10^{-5}$ M. Based on this profile, Xen2174 was developed with improved chemical stability and duration of efficacy in animal models of pain (Nielsen et al., 2005). After extensive preclinical toxicology, Xenome Ltd evaluated Xen2174 intrathecally in a Phase I/IIa safety trial in cancer patients suffering poorly managed pain. Initial results have proved promising both in terms of safety and efficacy. Thus, selective targeting of spinal NET may offer advantages over existing spinal therapies for pain control.

4. **Redox modulation of T-type calcium channels**

As outlined earlier, Ca$_{v}$3.2 T-type VGCCs are critical mediators of the excitability of primary afferent neurons. The activities of these channels are regulated by a number of cellular mechanisms, including redox modulation. For example, reducing agents such as dithiothreitol (DTT) and L-cysteine (L-cys) selectively enhance low-voltage-activated (T-type) calcium currents in acutely dissociated smaller dorsal root ganglion (DRG) neurons, most of which are nociceptors (Todorovic et al., 2001). Furthermore, L-cys, DTT and other thiol-containing analogues of L-cys produce thermal and mechanical pain sensitization when injected into the peripheral receptive fields of these neurons in vivo (Todorovic et al., 2001; Pathirathna et al., 2006). It has also been reported that reducing agents enhance neuronal excitability in a unique subpopulation of IB4-positive nociceptors, termed “T-rich” cells, that expresses high density of T-type currents and virtually no high-voltage-activated (HVA) Ca$^{2+}$ currents (Nelson et al., 2005). In current clamp recordings, reducing agents selectively sensitize classical C-type nociceptors with wide action potentials that express T-type currents. This is manifested as a reduced rheobase and increased probability of membrane firing in the presence of physiologically-relevant concentrations of L-cys (Nelson et al., 2007). Furthermore, exogenous (e.g., tricine) and endogenous (e.g., albumin) agents capable of chelating zinc ions mimic and occlude the effects of L-cys and DTT on the amplitude of T-type current and T channel-dependent cellular excitability of DRG cells. In contrast, cysteine-modifying agents such as N-ethylmaleimide (NEM) or UV light do not prevent the effects of reducing agents on T-current in DRG cells. Site directed mutagenesis of Ca$_{v}$3.2 revealed that a single-point mutation of Histidine 191 to glutamine completely abolishes sensitivity of Ca$_{v}$3.2 to both reducing and chelating agents, without any adverse effect on channel kinetics per se. Furthermore, the reverse mutation in the Ca$_{v}$3.1 T-type channel subtype was able to confer L-cys and DTT sensitivity to the previously completely redox-insensitive Ca$_{v}$3.1. In addition, H191Q mutation disrupted high-affinity zinc inhibition of Ca$_{v}$3.2, as manifested with a 40-fold increase in IC$_{50}$ for zinc blockade of Ca$_{v}$3.2 currents, suggesting that the effects of reducing agents may occur indirectly via disinhibition of the channels by endogenous zinc ions. Reducing agents, as well as synthetic and endogenous chelators of zinc, sensitize Ca$_{v}$3.2 T-type current-containing nociceptors isolated from wild-type mice, but not nociceptors from Ca$_{v}$3.2 knockout mice, thus underscoring the potential importance of redox/zinc regulation of T-type channels in the context of pain. Similar mechanism likely exists *in vivo* since L-cys injected intradermally into hind paws induced profound thermal hyperalgesia in wild-type but not Ca$_{v}$3.2 knockout mice. Because the majority of C-type nociceptors are polymodal, this mechanism of sensitization may be relevant to a variety of pain conditions involving exposure to acute noxious thermal, mechanical, and chemical stimuli. Further, the elucidation of the molecular mechanisms underlying the role of T-type channels in peripheral sensitization of pain responses may offer insight into opportunities for new targets in analgesic pharmacotherapy. Future experiments will address the issue of the pathological conditions that may involve abnormalities of zinc interaction with Ca$_{v}$3.2 in peripheral nociceptors.

5. **T-type calcium channels as targets for small molecule analgesics**

Interest in developing clinical T-type VGCC inhibitors as a therapy for neuropathic and chronic pain conditions arises from a common theme found in different pain states: the persistent, spontaneous and repetitive activation of the primary afferent neurons (Devor et al., 1994; Kajander and Bennett, 1992; Petersen et al., 1996; Song et al., 1999; Zhang et al., 1999). As outlined above, several lines of evidence implicate the Ca$_{v}$3.2 subtype of the T-type VGCC family in the pathophysiology of neuropathic and inflammatory pain. While there is no selective pharmacologic inhibitor of Ca$_{v}$3.2 channels currently available, prototypical non-selective VGCC inhibitors with T-type channel blockade (e.g., ethosuximide) attenuate dorsal horn neuronal responses in rats (Matthews and Dickenson, 2001). Compounds with modest peripheral T-type channel inhibition (e.g., mibebradil) reverse experimental neuropathic pain (Dogrul et al., 2003), are anti-hyperalgesic (Barton et al., 2005) and effective in models of paclitaxel and vincristine-induced peripheral neuropathy (Flatters and Bennett, 2004). Neuromed’s discovery program has developed a series of small organic compounds that support the hypothesis that Ca$_{v}$3.2 selective VGCC blockers are sufficient to mediate pain relief, including thermal hyperalgesia produced by the inflammmogen carrageenan, with efficacy closely paralleling systemic exposure levels.

Based on the physicochemical properties of existing T-type channel inhibitors, we would predict that that compounds that do not readily enter the brain (i.e., ‘peripherally acting’) could have broad spectrum effects on acute and inflammatory pain. It also appears likely that compounds with mixed T- /N-type VGCC blocking activity should be highly efficacious in mediating full spectrum pain relief across a wide range of neuropathic pain dimensions. Finally, compounds with N-
type and T-type blocking actions show some interesting potential as abuse deterrents for alcohol abuse [Newton et al., 2004, 2008]. If extended to other mechanisms of substance abuse (such as the opioids), centrally acting T- and N-type VGCC blockers may prove to be powerful new mechanism to treat moderate to severe pain states without the abuse potential known for opioids.

6. Summary
Voltage gated calcium channels continue to be major areas of focus in the development of new therapeutic approaches for the treatment of chronic pain. This may not only include the identification of novel channel blocking molecules, but also an exploitation of intrinsic regulatory mechanisms that control the activities of both N- and T-type calcium channels to increase efficacy and limit side effects.

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