Challenging the catechism of therapeutics for chronic neuropathic pain: targeting CaV2.2 interactions with CRMP2 peptides

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Abstract

Chronic neuropathic pain management is a worldwide concern. Pharmaceutical companies globally have historically targeted ion channels as the therapeutic catechism with many blockbuster successes. Remarkably, no new pain therapeutic has been approved by European or American regulatory agencies over the last decade. This article will provide an overview of an alternative approach to ion channel drug discovery: targeting regulators of ion channels, specifically focusing on voltage-gated calcium channels. We will highlight the discovery of an anti-nociceptive peptide derived from a novel calcium channel interacting partner – the collapsin response mediator protein 2 (CRMP2). *In vivo* administration of this peptide reduces pain behavior in a number of models of neuropathic pain without affecting sympathetic-associated cardiovascular activity, memory retrieval, sensorimotor function, or depression. A CRMP2-derived peptide analgesic, with restricted access to the CNS, represents a completely novel approach to the treatment of severe pain with an improved safety profile. As peptides now represent one of the fastest growing classes of new drugs, it is expected that peptide targeting of protein interactions within the calcium channel complex may be a paradigm shift in ion channel drug discovery.

1. Introduction

The Institute of Medicine of the National Academies in 2011 estimated that ~116 million adults in the USA (~1 in 3) suffer from chronic pain every year [50]. Chronic pain costs the nation up to $635 billion each year in medical treatment and lost productivity. Although some chronic pain conditions can be treated adequately with existing drugs, a large number of patients fail to achieve adequate pain relief, even with polypharmacy. Furthermore, currently available opioid pain therapies, which are generally only partially effective, are

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often associated with many side effects that limit their clinical efficacy, including tolerance and addiction. Hence, new research and therapies are critically needed to decipher pain mechanisms and open new avenues for specific and more effective treatments. Several excellent reviews describing the roles of voltage-gated calcium [94, 100, 113, 115] and sodium [36, 37] channels in pain have appeared in the literature; thus, in this review, we will focus on a new peptide-based therapeutic approach for chronic pain.

2. Rationale for targeting calcium channels for pain research

N-type voltage-gated calcium channels (CaV2.2) are multiprotein complexes comprised of a pore-forming α-subunits and auxiliary α2/δ, β, and γ subunits [39, 102]. CaV2.2 channels are localized to primary afferent terminals in laminae 1 and 2 of the dorsal horn [108] where their activation results in the influx of calcium and release of neurotransmitters such as glutamate, substance P, and calcitonin gene related peptide (CGRP). Following discovery of CaV2.2-specific conotoxins, the biological role of this channel was evaluated in animals through the use of isoform-specific inhibitors. Specific interest in the role of CaV2.2 in sensation arose from the observation that omega-conotoxin (ω-CTX) was able to block transmitter release from sensory neurons and spinal nerve terminals [46, 61]. CaV2.2 channels are also critical for pain transduction as block of these channels relieves hyperalgesia [8, 47, 56], mice lacking CaV2.2 show an increased threshold for pain [84], and expression of CaV2.2 is upregulated following a chronic constrictive nerve injury [34]. Spinal administration of CaV2.2 blocking peptides has been shown to ameliorate painful behavior in rodent models of neuropathic and inflammatory pain [87]. Interestingly, several CNS effects were also observed in the mice including; decreased anxiety behavior, changes in vigilance, and enhanced aggressive behavior [15, 55, 84]. The importance of CaV2.2 in pain is further underscored by the demonstration of a naturally occurring alternative splice form of CaV2.2 (i.e. exon 37a) in small-diameter nociceptive neurons [14] which are critical for basal thermal nociception, and thermal and mechanical hyperalgesia [8]. The role of CaV2.2 in neurotransmitter release and pain sensation is further reinforced by studies demonstrating that analgesic opioids and adrenergic agonists inhibit CaV2.2 [89]. In sum, by virtue of their ability to control the regulated release of neurotransmitters from nociceptive afferents, N-type Ca^{2+} channels are a prime target for the development of novel analgesics [86, 94, 113, 115].

3. Examples, limitations, and pipeline of CaV2.2-targeted drugs

Several subtypes of neuronal voltage-gated calcium channels are expressed in the nociceptive pathway and are crucial for shaping action potentials and controlling cellular excitability and synaptic transmission [115]. Recently, Ziconitide (trade-name Prialt®) was approved by the Food and Drug Administration (FDA) for the treatment of severe pain that was refractory to other therapies [38]. This drug is a synthetic version of the naturally occurring cone snail toxin μ-conotoxin MVIIA and is a highly potent and selective peptide blocker of CaV2.2, validating N-type calcium channels as a promising target of novel analgesics. However, due to the profound side effects and a lack of efficacy when delivered by more common routes (i.e. intravenous or oral), the use of Prialt® is limited to intrathecal pump route of administration, i.e. direct application to the spinal synapse between sensory neurons and spinal neurons. That intrathecal application is required and sufficient for analgesia also supports the notion that presynaptic blockade of CaV2.2 is responsible for targeting pain. The use of Prialt® via this route is not without complications as the intrathecal route of administration is by far the most expensive and invasive method of drug delivery. Many side effects are accompanied by the use of the drug that often manifest as confusion, depression, hallucinations, decreased alertness, somnolence, orthostatic hypotension and nausea [79, 85, 92, 99]. Prialt® is also contraindicated in patients with pre-

existing mental disorders due to thoughts of suicide and worsening depression or paranoia [62]. Subsequently, Prialt® therapy in practice for neuropathic pain treatment, is warranted for relatively few patients who are intolerant or refractory to other treatments [109], such as systemic analgesics, adjunctive therapies or intrathecal morphine [6, 76, 77]. Prialt® is a state-independent CaV2.2 blocker and therefore inhibits the activity of the channel in both the hyperpolarized state (closed) and the depolarized state (open or inactivated) [42]. Thus, the side effects presumably occur through a non-selective blockade of N-channels in all tissues [1, 98].

Despite the shortcomings of Prialt®, blockade of the presynaptic, CaV2.2 and its affiliated subunits remains an important therapeutic target for a number of chronic pain conditions [39, 94]. One commonly used drug that targets the $\alpha 2\delta 1$ subunit of CaV2.2 is the antiepileptic drug, gabapentin (Neurotin®). Neurotin® is FDA-approved for a number of conditions including postherpetic neuralgia, diabetic neuropathy [12, 33, 45, 68], neuropathic pain [3, 18, 88], phantom limb pain [9, 16, 53, 71, 83], fibromyalgia [116] and trigeminal neuralgia [67, 74]. Consistent, though less compelling clinical evidence supports its use for a variety of other neuropathic pain syndromes, including cancer pain syndromes, pain associated with HIV infection and chronic back pain. Controlled clinical trials in diabetic neuropathy and postherpetic neuralgia [82] demonstrated that gabapentin at 2400-3600 mg/day has an efficacy similar to the tricyclic antidepressants [2]. Though gabapentin has a favorable pharmacokinetic profile, it is only available in oral form and requires at least three times daily dosing in order to achieve mean plasma levels throughout a 24-hour period to achieve efficacy [13]. However, one benefit of the drug is that, unlike Prialt, it does not require laboratory monitoring [103, 104].

Neurotin® is associated with only moderate to substantial benefits of pain relief in 31-43% of patients [67]. Adverse events are experienced by about two-thirds of people taking gabapentin, mainly dizziness, somnolence (27%), dizziness (24%), and ataxia (7%) and it is not an uncommon finding that patients are not able to tolerate dosing in the therapeutic range due to common side effects [67]. Gabapentin was also reported to disrupt the interaction between $\alpha_2\delta$-1 and thrombospondins *in vitro* and, as a result, interferes with synaptogenesis, although it does not affect pre-formed synapses [41], a finding that could encumber prolonged use of this class of drug.

There remains strong interest in the pharmaceutical and academic worlds toward the development of orally acting, state-dependent blockers of CaV2.2. Several research and development groups have made substantial progress in developing small organic molecules targeting 'hot spots' of the N-type calcium channel. One group in particular led, by scientific investigator Terrance P. Snutch, has targeted chemical moieties that possess calcium channel blocking activity, such as the diphenylmethylpiperazine basic skeleton [17, 72, 73, 114]. Through extensive structure-activity relationship (SAR) investigations two small organic molecules NP118809 and NP078585 have been discovered and optimized as a N-type calcium channel blockers with good selectivity over L-type calcium channels [114]. In doing so, Snutch and colleagues have gone on to commercialize several of these small molecules selective for N-type or T-type calcium channel blockers, two of which are steadily progressing through the FDA regulatory pathway by Zalicus, Inc, a biopharmaceutical company that discovers and develops novel treatments for patients suffering from pain. Specifically, Zalicus, Inc. is developing an orally available selective N-type calcium channel blocker (Z160) for the treatment of chronic neuropathic pain associated with lumbosacral radiculopathy and post-herpetic neuralgia. Z160 is in currently being tested in Phase II clinical trials (www.zalicus.com). Convergence Pharmaceuticals, LLC has also developed an orally available small molecule (CNV2197944) state-dependent N-type calcium channel blocker for the treatment of neuropathic pain, currently in Phase IIa clinical trials

(www.convergencepharma.com). Newron Pharmaceuticals SpA is perhaps the furthest along the pipeline, as their small molecule drug Ralfinamide is in Phase IIB/III trials, and is believed to mediate its potent analgesic effect through the inhibition of sodium channels, including NaV1.7, N-type calcium channels and NMDA receptor for the treatment of lower back pain (www.newron.com). Small molecule equivalents of omega-conotoxin CVID (e.g. AM336/CNSB004/Leconotide), the selective N-type calcium channel blocker, have also been engineered and are being tested in early stage preclinical studies for pain associated with diabetes and cancer [58, 59, 93].

In addition to direct block of calcium channels, targeting of protein-protein interaction (PPI) interfaces within a PPI complex is gaining momentum as a potentially new manner of drug development [27]. Several small molecule PPI inhibitors are currently in clinical development by pharmaceutical companies Abbott, Genentech, Johnson & Johnson, or Roche; two drugs are already on the market (Tirofiban/Aggrastat® and Maraviroc/ Selzentry®) [44]. The notion of PPIs altering CaV2.2 channel function was proposed almost two decades ago by William Catterall and colleagues who discovered that interruption of the CaV2.2-syntaxin interaction with SYNaptic Protein INTeraction (synprint) peptides inhibits fast, synchronous transmitter release [66, 80, 90, 91]. Despite this early report, these patented [28] peptides have not advanced further. Additional peptides that attenuate G protein modulation of Ca^{2+} channels have also been reported and may be of use in probing calcium channel mediated presynaptic mechanisms [25, 26]; however, none of these peptides have been translated beyond. As will be described in the following sections, Sophia Therapeutics, LLC in collaboration with Indiana University has developed a novel peptide platform that target CaV2.2 by modulating channel activity through protein/protein interactions for the treatment of neuropathic pain. Sophia Therapeutics is currently performing preclinical *in vivo* studies and is actively seeking funding and partnership opportunities to move the project into Phase I clinical trials.

4. Discovery of CRMP2, a novel regulator of CaV2.2

Alternative strategies that target proteins interacting with calcium channels represent an emerging theme in drug discovery for treatment of clinical neuropathic pain. A proteomic screen identified the collapsin response mediator protein 2 (CRMP2) as a putative interacting partner of CaV2.2 [54, 69]. Enriched at functional release sites, CaV2.2 forms part of a large macromolecular complex, which facilitates efficient neurotransmitter release. Identification and analyses of a litany of protein-protein interactions within the nerve terminal have demonstrated a functional coupling between presynaptic Ca^{2+} channels and the transmitter release machinery [31, 35, 57, 95]. Although neurotransmission was not affected in CRMP1 knockout mice [97], long-term potentiation, spatial learning and memory were affected in both CRMP1 and CRMP3 knockout mice [78, 97], suggesting a possible role for CRMPs in neurotransmission as well as synaptogenesis. As potential binding partners, CRMPs could affect neurotransmission by either a physical interaction with the synaptic machinery or CaV2.2 itself.

Our study showed that CRMP2 is part of the CaV2.2 proteome [22, 32, 54]. Immunocytochemistry revealed a strong colocalization between CRMP2 and CaV2.2 within hippocampal neurons that was supported by reciprocal co-immunoprecipitation of the two proteins. CRMP2 localized to both extrasynaptic and synaptic fractions, suggesting that it may traffic CaV2.2 within these regions (Figure 1). CRMP2 was present in both cytosolic and membrane fractions. CRMP2 was found to localize to the soma, dendrites, and axons and therefore may interact with CaV2.2 in any of these regions. Traditional *in vitro* binding experiments [22] and isothermal titration calorimetric analyses (Khanna, M. and Khanna, R.; unpublished data) mapped the interaction to two domains within the cytoplasmic loops of

CaV2.2 [22]. The CRMP2–CaV2.2 interaction was dynamic as potassium chloride-induced depolarization led to an increase in the interaction. Functionally, the interactions led to an increased cell-surface expression of CaV2.2 that caused an increase in CaV2.2 current density in hippocampal neurons. This CRMP-2–mediated increase in Ca^{2+} influx translated into an increase in the release of excitatory neurotransmitter glutamate; toxin block of CaV2.2 eliminated this increase. CRMP2 overexpression also increased stimulated release of CGRP from dorsal root ganglia (DRG) [32]. Furthermore, knockdown of CRMP2 dramatically reduces Ca^{2+} currents and transmitter release [32]. These findings suggested that the biochemical interaction between CRMP2 and CaV2.2 was required for proper channel trafficking and function.

The strong effect of CRMP2 knockdown on Ca^{2+} currents in neurons suggested that CRMP2 is *necessary* for CaV2.2 function. Of significant interest, CaV2.2 current is observed in heterologous systems despite limited CRMP2 expression in these cells, which suggests that rather than directly enhancing CaV2.2 surface expression, CRMP2 may be dis-inhibiting a negative regulator of CaV2.2 trafficking. This negative regulator would likely be neuron specific, as CRMP2 expression appears to enhance surface expression in neurons, but not in heterologous systems (Priest and Khanna, personal communication). The negative regulation may also represent a channel-recycling pathway, which, without CRMP2, may lead to a majority of channels leaving the membrane and being degraded. As CRMP2 binds to the I-II loop, which has been shown to be an important region for proteasome regulation of CaV2.2, this could be a possible mechanism CRMP2's regulation of CaV2.2 [106]. Assuming that CRMP2 is responsible for overcoming neuronal specific negative regulators of CaV2.2 would also suggest a mechanism distinct from the other CaV2.2 subunits. Future work will be necessary to further explore the precise mechanism of CRMP2's regulation of CaV2.2.

5. Design, mechanism of action, and characterization of a CRMP2 peptide as anti-nociceptive

Having established CRMP2 as a *bona fide* regulator of CaV2.2 function, we reasoned that disruption of this biochemical and functional interaction may not only lead to the development of a useful tool for studying CaV2.2 channel trafficking but also may yield a potential therapeutic for pain as suppression of CaV2.2 channel activity had previously been reported to be anti-nociceptive [113]. Our initial mapping of the CaV2.2:CRMP2 interaction had identified a surface-exposed region in CRMP2, which we designated calcium channel binding domain 3 (CBD3) [22]. Further mapping of CaV2.2 binding to overlapping peptides encompassing the full length of CRMP2 yielded four peptides with significant binding to CaV2.2 [21]. Remarkably, of these four, the CBD3 peptide identified in our earlier study displayed the highest binding to CaV2.2. The sequence of this fifteen amino acid peptide is relatively conserved amongst CRMPs 1, 3, 4 and 5 with CRMP3 displaying the highest homology (12 of 15 residues are identical). Interestingly, the CBD3 peptide sequence is completely conserved from humans to zebrafish. The CBD3 peptide bound to immobilized loop 1 and the distal part of the carboxyl terminus of CaV2.2 and in a concentration-dependent fashion, blocked the interaction between recombinantly purified CRMP2 and loop regions of CaV2.2, between full-length native CaV2.2 and recombinantly purified CRMP2, demonstrating usefulness as a tool for interrupting the interaction between this protein complex. Tubulin-CRMP2 interaction or the CaV2.2-syntaxin interactions were not affected by CBD3, demonstrating specificity. CBD3 appears to have both an acute as well as a long-term effect on CaV2.2 function. When introduced into heterologous cells, the CBD3 peptide reduced calcium currents likely by preventing surface trafficking of CaV2.2 or increasing CaV2.2 internalization. When CBD3 was expressed in hippocampal neurons, it

antagonized CRMP2-induced enhancement of Ca^{2+} channel currents. As CBD3 disrupts the interaction between CRMP2 and CaV2.2, this supports the conclusion that the interaction between CRMP2 and CaV2.2 is likely responsible for the observed increase in Ca^{2+} currents.

Subsequent creation of TAT-CBD3 (tagged with the HIV-1 transactivator of transcription (TAT) cell penetrating motif) allowed evaluation of this peptide directly in cells as well as in animal models of pain. TAT-CBD3 peptide interfered with CRMP2-CaV2.2 interactions resulting in acute inhibition of CaV2.2 currents in sensory and hippocampal neurons; acute inhibition of frequency of spontaneous excitatory postsynaptic currents (sEPSCs) in spinal cord slices as well as layer V pyramidal neurons suggesting reduction in probability of glutamate release from stimulated presynaptic terminals; and inhibition of evoked calcitonin gene-related peptide (CGRP) in sensory neurons in culture (acute and long-term inhibition observed) and in spinal cord slices.

Our results converged on the possibility that TAT-CBD3 might act as a novel antagonist of CaV2.2 function prompting us to investigate its usefulness as a therapeutic for pain using an *in vivo* model of hypersensitivity. TAT-CBD3 was tested in a battery of behavioral assays including formalin-induced nocifensive behavior, capsaicin-induced nocifensive behavior, and chronic neuropathic models including distal sensory polyneuropathy induced by anti-retroviral drugs, lysophosphotidylcholine-induced sciatic nerve focal demyelination (LPC), and traumatic tibial nerve injury model. In all of these models, TAT-CBD3 reduced nocifensive behaviors or induced reversal of hypersensitivity. Notably, these effects were observed with systemic injections of TAT-CBD3 in stark contrast to the intrathecal route of delivery espoused for Prialt ®.

Importantly, in a further string of rodent behavioral tests to examine off-target effects, TAT-CBD3 was mildly anxiolytic without affecting memory retrieval, sensorimotor function, or depression at doses at least 50 fold higher than needed to achieve anti-nociception. Sympathetic-associated cardiovascular activity was also not affected by TAT-CBD3 [110]. Thus, peptide analgesics, such as TAT-CBD3, with restricted access to the CNS represent a completely novel approach to the treatment of severe pain with a likely improved safety profile.

6. CRMP2 peptides: good, better, best?

While we have documented great success with TAT-CBD3, challenges remain including the transiency (4 hours) of pain reversal with TAT-CBD3 [21], the lack of efficacy in the tibial nerve injury model of persistent neuropathic pain [110], coupled with lack of knowledge of the time course (i.e., pharmacokinetics/bioavailability) of TAT-CBD3's action, additional possible targets and a safety profile have not been fully investigated. In an effort to increase TAT-CBD3's duration of action and utility in a broader range of neuropathic conditions, we have converged our initial efforts on optimizing the CBD3 platform by selected single site mutagenesis and using alternative cell penetrating motifs.

To address the first issue, a peptide array harboring systematic amino acid scans of the CBD3 coupled with a Far-Western approach identified three peptides with point mutations at positions 6 (A6K), 9 (R9L) and 14 (G14F) with greater binding to Ca^{2+} channels than the parent CBD3 peptide [75]. One of these peptides, TAT-CBD3-A6K, was modeled as an α-helix and molecular dynamics (MD) simulations were performed to explore atomistic flexibility of wild type and A6K mutant peptides in solution. This analysis showed that like the wild type peptide, the C-terminus residues are less stable than N-terminus residues, which consistently maintain an α-helical structure throughout the MD simulation. Increased

conformational change of the mutant peptide may result in greater efficacy for this peptide. Greater conformational change suggests that the peptide may sample alternative conformational states that are more prone to bind to the calcium channel and inhibit its interaction with CRMP2. The wild type peptide appears more prone to undergo structural changes that are not seen in the A6K mutant simulations. Based on these structural predictions, we tested the mechanism of action and efficacy of TAT-CBD3-A6K and found that (i) this peptide exhibited greater anti-nociception in a rodent model of AIDS therapy-induced peripheral neuropathy when compared to the parent TAT-CBD3 peptide; (ii) intraperitoneal administration of TAT-CBD3A6K produced none of the minor side effects (i.e. tail kinking, body contortion) observed with the parent peptide; (iii) excitability of dissociated small diameter sensory neurons isolated from rats was also reduced by TAT-CBD3A6K; and (iv) suppression of excitability may be due to inhibition of T- and R-type Ca^{2+} channels (Figure 4). These results suggested that structural modifications of the CBD3 scaffold peptide might result in peptides with selectivity against a particular subclass of voltage-gated calcium channels resulting in a multi-pharmacology of action on the target.

To improve CBD3's utility to broader range of neuropathic conditions, CBD3 was grafted to a stretch of nine arginines ((R9)-CBD3); the cell penetrating peptide (CPP) R9 motif was chosen due to its superior cell penetrating abilities [107]: it is the most efficacious of currently known protein transduction domains with at least a 20–fold better penetrability into cells than TAT [107] or other homopolymeric amino acids [65]; it is well tolerated by cells, with low short- and long-term toxicological effects [101]; and its the mechanism of transduction into cells is well understood and involves binding to cell surface heparan sulfate proteoglycans, heparin sulfate-mediated endocytosis into vesicles, release of R9 from heparin sulfate upon cleavage by heparanases, culminating in release of unbound R9 into the cytosol due to vesicular leakage [43]. Three endocytic pathways – macropinocytosis, clathrin-mediated endocytosis and caveolae/lipid-raft-mediated endocytosis – are thought to be involved [40]. Similar to TAT-CBD, the R9-conjugated CBD3 peptide interfered with the CaV2.2–CRMP2 interaction. The interference was specific, as the tubulin-CRMP2 interaction was not disrupted by R9-CBD3. The functional consequence of the disrupted CaV2.2–CRMP2 interaction was a significantly higher extent (i.e. efficacy) of inhibition of calcium influx in sensory neurons. This increased efficacy may possibly contribute to the mechanism of action of this peptide. That R9-CBD3 did not affect Ca^{2+} influx activated by vanilloid receptor activators resiniferatoxin and capsaicin rules out targeting of these receptors as a potential mechanism of action. These results are entirely consistent with our previous data that showed no effect of TAT-CBD3 on TRPV1 current recordings following capsaicin challenge [21] demonstrating that TAT-CBD3 does not work through direct inhibition of TRPV1 channels. We found that R9-CBD3, like TAT-CBD3 [21], was effective at reversing mechanical hypersensitivity induced by NRTI-associated distal symmetrical polyneuropathy. Importantly, R9-CBD3 reversed mechanical hypersensitivity associated with nerve-injury possibly due to its superior cell transduction attributes [107] (Figure 5).

Future efforts will explore alternative routes of dosing including continuous subcutaneous infusions, intramuscular, sublingual and or oral routes. Clinically, ~2-4 grams/day of gabapentin are needed to achieve significant reduction in average daily pain intensity scores compared with placebo [13]; but at this dosage the drug is not without significant side effects. Even taking into account allometric scaling, TAT-CBD3 reverses NRTI-induced hypersensitivity at 0.1 mg/kg compared with 200 mg/kg of gabapentin needed to achieve similar levels of reversal [21]. One therapeutic challenge for many neurologically acting medications, including TAT-CBD3, is penetration of the blood brain barrier (BBB). The peptide, like Prialt®, appears to have a somewhat restricted penetration into the CNS [21], however disruption of the BBB due to direct injury to the spinal cord/brain or disease

pathologies may allow the peptide to overcome the CNS's physicochemical barriers and alter synapse functioning within higher order systems [20]. More importantly, that peripheral nerve injury or inflammatory pain states may be maintained by CNS gliosis (activated glial cells that release inflammatory mediator) suggests that there could be chronic disruptions in BBB permeability and transport of TAT-CBD3 into the brain. So, despite the relatively restricted penetration across the BBB by TAT-CBD3 it could still have cognitive effects following chronic dosing. Thus, in the future we will also evaluate whether the peptides affect memory and learning, locomotion, social interaction as well as examine histopathological changes in various tissues.

7. Getting over the "small-molecule fever": peptide market, limitations and optimization

Peptide based therapeutics for pain and a number of other chronic conditions are on the rise. The number of peptides approved in the past few years falls third in the line with small molecules (45% of the total) and 'proteins' (24% of the total). Between 2009-2011, eight peptides were accepted by the FDA, which represents 11% of the total drugs accepted in this period. The probability of regulatory approval for peptides is over 20%, a rate which is double that of small molecules [60]. Although these peptides have a broad range of structures, size, and complexity, it has not limited their place in the market [5]. The peptide market consists of a relatively small portion of all drug product sales (~1.5%). However, peptide drug product sales are increasing with an annual growth of 7.5-10% [60]. Currently, there are 80 peptides on the market, approximately 200 more in clinical trials and 400 in advanced preclinical states [105], which have demonstrated diverse applications across a number of clinical fields [11, 96].

Nature continues to be the main source and best inspiration for the drug discovery process [70]. The clear advantage that drug peptides offer include, a significantly high structural relationship between physiological active parent molecules thereby reducing the risk of off-target reactions [60] and lack of immunogenicity [64]. Overall, peptides have several advantages as drug; including their high biological activity (given their natural source), high specificity, and low toxicity [5]. Peptides also have the ability to penetrate further into tissues due to their smaller size and require substantially less quantity to activate their target [48]. Compared to small organic molecules, peptides typically offer greater efficacy, selectivity and specificity [49].

In general, it is believed that peptides have several features that prevent active pharmaceutical ingredients; such as, lack of oral bioavailability, low stability under physiological conditions [24, 117], short half-life because of their rapid degradation by proteolytic enzymes of the digestive system, and hydrophobicity (limits their ability to cross physiological barriers) [5]. However, robust peptide chemistry strategies and 'modern' solution organic synthetic chemistry provide for straightforward analog analysis for lead-candidate optimization. The process is quite efficient and economical when compared with small molecules [5].

The half-life of many peptides is primarily dependent on their proteolytic susceptibility. Orally available peptides inevitably battle gram quantities of proteolytic activity in the lumen of the small intestine, which are secreted by the pancreas (e.g. alpha chymotrypsin, trypsin, pancreatic elastase, carboxypeptidases A, etc.). Additionally, the membrane of epithelial cells contains at least 15 peptidases. Other proteolyic sources include lysosomal peptidases and matrix metalloproteinases [105, 112]. Thus, peptide modifications have become necessary strategies to optimize their function as an ideal drug-delivery system and bypass many of the limitations associated with natural peptide kinetics. Chemical

optimization strategies are based on structure-activity relationship and/or quantitative structure-activity relationship; including cyclization of peptide sequences, substitution with an unnatural amino acids, isosteric amide bond replacement, N-terminal esterification (to enhance plasma stability), peptide conjugation with cell penetrating peptides (i.e. TAT conjugation) just to name a few [4, 105]. For example, Ahrens and co-workers developed 'intelligent peptides' which have smart linkers that increase specificity and blood plasma stability [4]. With the sophistication currently possible in peptide modifications, a promising future for innovative synthetic therapeutic peptides may provide the next generation of pharmaceutical drugs.

8. Conclusions

It is clear that CaV2.2 is a nidus for neurotransmitter release [10, 29, 30] and nociceptive transmission [115]. However, use of CaV2.2 blockers in pain therapeutics (Prialt® or gabapentin) is limited by side-effects resulting from inhibition of the physiological functions of CaV2.2 within the CNS. To circumvent this, we have advanced a strategy for targeting protein interactions that regulate voltage-gated calcium channels as an alternative to direct channel block. In this regard, we identified CRMP2 as a *bona fide* modulator of CaV channels[22, 32] with peptides from CRMP2 proving to be anti-nociceptive[21, 51, 81]. Remarkably, interrupting the CaV-CRMP2 interaction with a peptide derived from the CaV channel reversed pain behavior associated with peripheral neuropathy [111], further validating the CaV-CRMP2 axis as a novel node for development of pain therapeutics. Importantly, targeting channel regulation may potentially avert many of the adverse side effects associated with direct channel block. Indeed, the CRMP2-derived TAT-CBD3 peptide has a problem-free profile with at least a 500-fold therapeutic window [21]. That replacement of a lysine with an alanine residue at the sixth residue of CBD3 allowed the resulting peptide (TAT-CBD3-A6K) to reverse tibial nerve-injury-induced pain which was refractory to TAT-CBD3 [110], as well as bestowed upon the new peptide an ability to block T- and R-type calcium channels (Figure 4) suggests opportunities to tailor peptides for specific pain indications and will be addressed in the future. In conclusion, the TAT-CBD3 peptide is efficacious in both acute and chronic pain models when administered either topically or systemically; CBD3, interfering with the function of CaV2.2, produced analgesic effects in rodents subjected to formalin-induced nocifensive behavior, capsaicin-induced nocifensive responses to ocular administration and neurogenic inflammation of the meninges, and reversed the chronic tactile hypernociceptive behavior observed in antiretroviral toxic neuropathy. Collectively, the antinociceptive activity of TAT-CBD3 across a number of pain models suggests that N-type voltage calcium channels on the presynaptic terminals of afferent sensory neurons play a central role in both inflammatory and neuropathic pain behaviors.

Acknowledgments

Abbreviations

CGRP	calcitonin gene related peptide
CaV2.2	N-type voltage-gated Ca^{2+} channel
CBD	Ca^{2+} channel binding domain
CRMP2	collapsin response mediator protein 2
CPP	cell penetrating peptide
CNS	central nervous system
ω-CTX	omega-conotoxin
DIV	days in vitro
DRG	dorsal root ganglion
PWT	paw withdrawal threshold
R9	nona-arginine
ST	Sophia Therapeutics
TAT	HIV-1 transactivator of transcription domain
TNI	tibial nerve injury
CTX	conotoxin
LPC	lysophosphotidylcholine
sEPSCs	spontaneous excitatory postsynaptic currents
NRTI	nucleoside reverse transcriptase inhibitor
BBB	blood brain barrier

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Highlights

- CaV2.2 calcium channels are a prime target for development of novel analgesics

- Targeting CaV2.2 complex proteins may avoid side-effects of CaV2.2-targeted drug

- Axonal growth protein CRMP2 interacts with and regulates function of CaV2.2

- CRMP2 peptide is anti-nociceptive in models of neuropathic pain

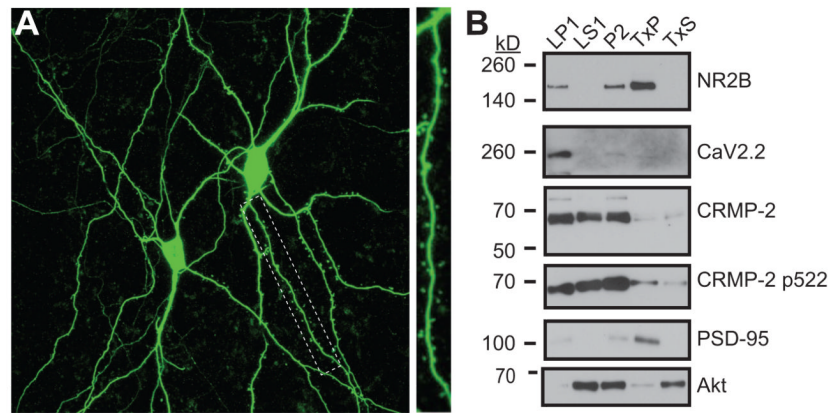- Tailored CRMP2 peptides hold promise for next generation analgesics

Figure 1. CRMP2 localization
(**A**) Representative pseudo-colored image of two CRMP-2-EGFP transfected hippocampal neurons in organotypic slice cultures 5 days post transfection. The expanded region shows a primary dendrite with robust expression in spines. Image provided by Dr. Xiaoming Jin. (**B**) Synaptic and extra-synaptic fractions from postnatal day 1 rats were isolated using differential centrifugation and detergent selective sedimentation and immunoblotted with antibodies as indicated. The post-synaptic density fraction (PSD) is insoluble in Triton X-100 and was used to separate synaptic (Triton X-100 insoluble pellet, TxP in which the proteins PSD-95 and the NMDAR are abundant) from extra-synaptic fractions (Triton X-100 soluble, TxS). Fractions and percent loaded were: LP1 (0.8%), intracellular membranes; LS1 (0.1%) cytosol non-membrane; P2 (0.3%) synaptosomes; TxP (1.2%) synaptic fraction; and TxS (0.4%) extra-synaptic. Molecular weight markers are indicated in kilodaltons (kD). Data are reproduced from the doctoral work of Dr. Brittain in the Khanna laboratory [19].
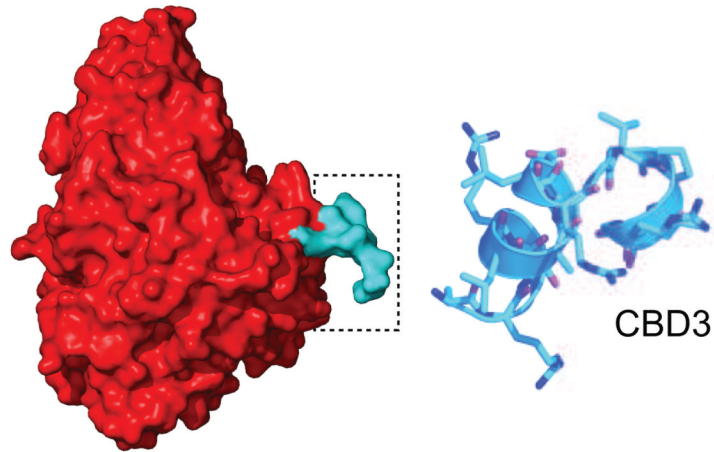
Figure 2. CRMP2 structure and CBD3 model
Crystal structure of momomeric CRMP2 [63]. As the CRMP2 structure is truncated at amino at 490, only 6 of the fifteen amino acids that comprise CBD3 are present in this structure. These 6 amino acids are shown in the boxed region in the crustal structure. A model of the entire fifteen amino acid CBD3 peptide [75] is shown in blue illustrating the plastic nature of the amino-terminal residues.
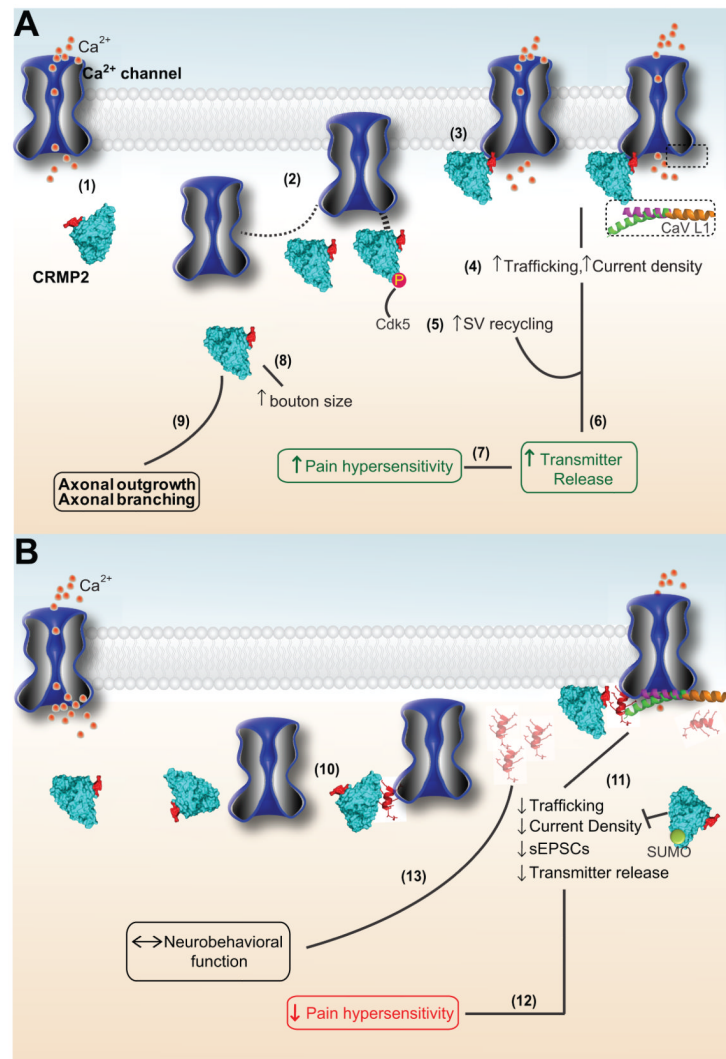
Figure 3. Targeting CRMP2/CaV2.2 interaction
(**A**) Functional consequences of CRMP2/CaV2.2 interaction. The cytosolic protein CRMP2 (1) interacts with the N-type calcium channel (2), increasing forward trafficking of CaV2.2 to the membrane (3). Phosphorylation of CRMP2 by the priming kinase cyclin dependent kinase 5 (Cdk5) increases the interaction between CRMP2 and CaV2.2 [23]. The increased surface expression directly correlates with increased calcium current density (4). Along with increased synaptic vesicle recycling (5), the increase in current density leads to a subsequent increase in neurotransmitter release (6) and, potentially, pain hypersensitivity (7). Additionally, CRMP-2 overexpression increases the average size of synaptic boutons (8), which may be attributed to its native functions in axonal outgrowth and branching (9). (**B**) TAT-CBD3 effectively blocks the interaction between CRMP-2 and CaV2.2 (10), leading to decreased membrane trafficking, current density, spontaneous EPSCs (sEPCs), and neurotransmitter release (11). Recent evidence suggests that post-translational modification of CRMP2 by the small ubiquitin-like modifier (SUMO) has a negative impact on calcium influx in sensory neurons [52]. The functional consequences of TAT-CBD3 treatment resulted in an overall decrease in pain hypersensitivity (12). Single doses of TAT-CBD3 have not demonstrated deleterious effects on neurobehavioral and sympathetic function (13).

Similar mechanistic effects on trafficking and overall *in vivo* effects on relief of chronic pain are observed with a peptide (structure shown [7]) from the calcium channel itself [111].
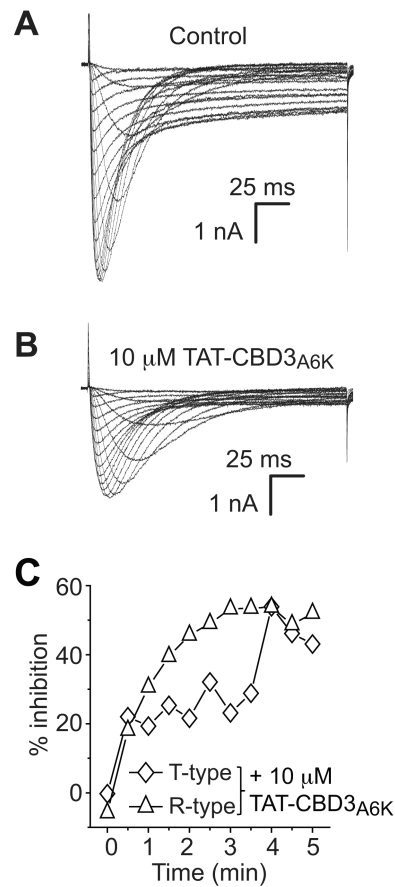
Figure 4. Effects of TAT-CBD3A6K on T- and R-type calcium currents in sensory neurons
Representative family of traces from a DRG neuron with both T- and R-type calcium currents before (**A**) and 5 min (**B**) after addition of 10 μM TAT-CBD3A6K. Currents were elicited in response to the 200 ms steps in 5 mV increments from −60 mV to +50 mV, from a holding potential of −90 mV. To isolate T- and R-type calcium currents, the extracellular bath solution contained 5 mM Nifedipine (Nif), 200 nM ω-Agatoxin IVA (Aga) and 500 nM ω-Conotoxin GVIA (CTX) to block L-, P/Q-, and N-type calcium currents, respectively. At −10 mV, T-type calcium currents contribute to >80% of the conductance with R-type contributing <3%. Time course of TAT-CBD3A6K mediated inhibition of T-type and R-type (**C**) calcium currents. Time course of inhibition is shown as averaged normalized current density (pA pF^{-1}) before peptide addition and at intervals of 30 s for 5 min. Averaged values are shown with standard error for 4–6 control cells and 4 cells following addition of 10 μM TAT-CBD3A6K. Some error bars are smaller than the symbols. Data represent mean ± SEM from n = 3–6 cells at each time point except for n = 2 the 4 min time point for T-type currents in the presence of peptide.
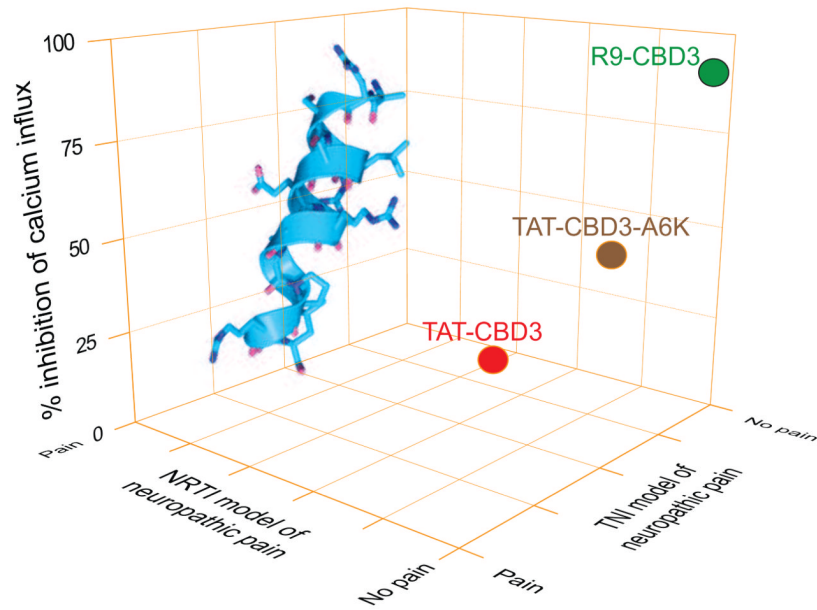
Figure 5. Tuning CBD3
Graph illustrating optimization of CBD3 with a single point mutant (A6K) or use of the nona-arginine cell-penetrating motif. The relative efficacy of each peptide in blocking depolarization-evoked calcium influx is shown. The efficacy of systemically administered peptides in the nucleoside reverse transcriptase inhibitor (NRTI)- or tibial nerve injury (TNI)-induced pain models is also shown.