

Novel coupling is painless

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The first report that several neurotransmitters, including γ -aminobutyric acid (GABA), decreased neuronal action potential duration in dorsal root ganglia (DRG) neurons appeared more than 35 years ago (Dunlap and Fischbach, 1978). Dunlap and Fischbach (1978) realized that the effects of GABA could not occur via the ionotropic GABA receptor—at that time the only known GABA receptor—and that the target ion channel was most likely a voltage-gated calcium (Ca_v) channel, rather than a Na_v or K_v channel. They correctly concluded that another type of GABA receptor must exist, which we now know is the G protein-coupled GABA type B (GABA_B) receptor. The modulated channel was later identified as the chick homologue of the N-type Ca^{2+} channel $\text{Ca}_v2.2$ (α_1B) (Cox and Dunlap, 1992), one of three members of the Ca_v2 family. GABA_B receptors in human and rodent sensory neurons and in various expression systems were shown subsequently to inhibit native N-current and recombinant $\text{Ca}_v2.2$ current, respectively (Raingo et al., 2007; Callaghan et al., 2008; Adams and Berecki, 2013). Inhibition primarily occurs by a voltage-dependent mechanism common to various neurotransmitters whereby $\text{G}\beta\gamma$ binds to $\text{Ca}_v2.2$ slowing channel opening, whereas positive voltage steps relieve this inhibition (Marchetti et al., 1986). The closely related P/Q-type (α_1A) channel, $\text{Ca}_v2.1$, exhibits similar modulation by GABA (Mintz and Bean, 1993). The third member of the Ca_v2 family, $\text{Ca}_v2.3$ (α_1E), is less susceptible to direct $\text{G}\beta\gamma$ modulation than the other two family members (Shekter et al., 1997). The revelation that mice with a deletion in either $\text{Ca}_v2.2$ or in $\text{Ca}_v2.3$ exhibited reduced neuropathic pain-like behavior, indicating that these channels participate in pain sensation signaling (Saegusa et al., 2000, 2001), sparked great interest in the regulation of Ca_v2 inhibition by GABA_B receptors in DRG neurons. Astonishingly, however, the precise mechanism of GABA_B receptor modulation of $\text{Ca}_v2.3$ channels has remained ill defined.

Given $\text{Ca}_v2.3$'s functional importance in pain pathways, the uncertainty surrounding whether GABA_B receptors modulate $\text{Ca}_v2.3$ seems remarkable. Several observations may provide insights as to why this question still awaits an answer. In neurons, native $\text{Ca}_v2.3$ is

referred to as R-type current, which is poorly defined as the current remaining after blocking the activity of T channels (Ca_v3) with nickel, L channels (Ca_v1) with dihydropyridines, and N, P, and Q channels with ω -conotoxins. Because selective and complete pharmacological blockade of any target rarely occurs, doubt has surrounded the notion that native neuronal “R-type” current arises from a pure population of $\text{Ca}_v2.3$ channels (Wilson et al., 2000; Yang and Stephens, 2009); $\text{Ca}_v2.3$ activation occurs over a range of relatively negative voltages (approximately -40 to -50 mV), which has been used as an additional defining characteristic; however, this activation profile overlaps with that of other Ca_v currents (Williams et al., 1994). A further confounding issue is that R current often contributes a small percentage of the total whole cell Ca_v current, making it difficult to measure its modulation accurately. In any event, the GABA_B receptor's ability to modulate R-type current has not been adequately examined in neurons, nor has its ability to modulate $\text{Ca}_v2.3$ been tested in a recombinant system.

In searching for novel treatments of neuropathic pain, in this issue Berecki et al. have now answered the long-languishing question of whether GABA_B receptors modulate $\text{Ca}_v2.3$ activity. In so doing, they have advanced three distinct research fields: (1) development of synthetic forms of naturally occurring toxins for treatment of neuropathic pain, (2) Ca_v current modulation, and (3) GABA_B receptor function. They demonstrate that cyclized Vc1.1 (cVc1.1), an orally active compound, derived by the cyclization of the synthetic α -conotoxin peptide Vc1.1 (Clark et al., 2010), selectively activates GABA_B receptors to inhibit recombinant $\text{Ca}_v2.3$ activity. $\text{Ca}_v2.3$ inhibition occurs by a voltage-independent, pertussis toxin (PTX)-sensitive mechanism that requires c-src kinase, a nonreceptor tyrosine kinase (see Okada, 2012), and channel phosphorylation in its proximal C terminus. The signaling pathway is similar to one that mediates voltage-independent inhibition of the $\text{Ca}_v2.2e[37a]$ splice variant observed in a subpopulation of nociceptive DRG neurons after GABA_B receptor stimulation (Bell et al., 2004; Raingo et al., 2007). In characterizing

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this mechanism, the authors identify cVc1.1's inhibition of $\text{Ca}_v2.3$ activity via GABA_B receptor activation as a potential therapeutic strategy for treating certain forms of neuropathic pain.

Pain sensation is complicated. Initially, pain is perceived by nociceptive sensory neurons whose cell bodies reside in DRG (see Costigan et al., 2009; Woolf, 2010). These neurons project to lamina I and II in the dorsal horn to trans-synaptically stimulate ascending spinal neurons. The signal then travels to the thalamus where sensory information is distributed to higher cortical areas. Nociceptive pain is characterized as a high threshold pain activated by immediate, intense stimuli such as noxious heat or a sharp prick. If an injury is sustained, inflammatory pain will occur, which is characterized by hypersensitivity or tenderness from an immune response in the area of an injury that may last for days but usually is reversible. Inflammatory pain, like nociceptive pain, is protective because it serves to discourage use and promote healing. In contrast, neuropathic pain, a disease state of the nervous system (Woolf, 2010), is maladaptive in that pain sensation remains despite the disappearance of the original insult. Often neurons sustain an injury such that the pain threshold decreases so that innocuous stimuli are perceived as painful and are characterized by increased intensity and duration. These changes may persist or become irreversible (Costigan et al., 2009).

Neuropathic pain is remarkably difficult to treat perhaps because of its varied quality, intensity, and source (Costigan et al., 2009). Not surprisingly, several ion channels have been implicated in mediating pain including Na_v , Ca_v , HCN, TRPV1, and various K^+ channels, NMDA receptors, and nicotinic receptors (Raouf et al., 2010). Therapeutically targeting any one of these channels to produce analgesia has undesirable side effects because of their widespread distribution both peripherally and centrally. Moreover, developing highly selective channel ligands is difficult, and their off-target binding contributes to further unwanted actions. Lastly, once a promising drug is identified, delivering it to its site of action in patients is problematic because of high biodegradability within and low cellular uptake through the gut, difficulty crossing the blood-brain barrier, and short half-life (Adams and Berecki, 2013). An example of the promises and difficulties of developing effective treatment for pain is the Conus snail peptide toxin MVIIA, a highly selective antagonist of $\text{Ca}_v2.2$ activity with FDA approval for use as a non-opiate analgesic and marketed under several names (SNX-111, Prialt, Ziconotide). By blocking $\text{Ca}_v2.2$ activity, MVIIA reduces transmitter release from nociceptive nerve endings (McGivern, 2006). However MVIIA must be injected intrathecally for treating neuropathic pain and has a narrow therapeutic range.

Despite limitations in using toxins to treat neuropathic pain, David Adams' laboratory in collaboration

with many colleagues has synthesized various native and modified Conus snail toxins and tested them for analgesic actions (Clark et al., 2010; Adams and Berecki, 2013). Vc1.1 is a 16-amino acid peptide, originally isolated and purified from *Conus victoriae* venom, but with the native posttranslational modifications of the toxin removed. It is classified as a cholinergic α -conotoxin based on sequence homology and its pattern of disulfide bridges (Sandall et al., 2003). Vc1.1 selectively antagonizes neuronal $\alpha 9\alpha 10$ nicotinic ACh receptor functioning. In vivo, Vc1.1 acts as an effective and long-lasting analgesic in the chronic constriction injury and partial nerve ligation rat models of human neuropathic pain (Sandall et al., 2003; Satkunathan et al., 2005). It also accelerates recovery from nerve damage in these two models. When Adams and colleagues tested Vc1.1, they found what seemed to be a second, more potent action of Vc1.1. Vc1.1 appeared to selectively inhibit high threshold (HVA) but not low threshold Ca_v currents in DRG neurons. At that time, only one other α -conotoxin, Rg1A, was known to inhibit HVA Ca_v current in DRG neurons; native, reduced, or alkylated forms of Vc1.1 as well as five other α -conotoxins exhibited no inhibitory action on Ca_v currents. When $\text{Ca}_v2.2$ activity was blocked with the selective ω -conotoxin CVID, Vc1.1 lost its inhibitory action on DRG neuron HVA Ca_v currents, identifying this modulated HVA Ca_v current as N current (Callaghan et al., 2008).

Further characterization of Vc1.1's actions on DRG neurons revealed that N-current inhibition by Vc1.1 occurred independently of rises in intracellular Ca^{2+} , but instead depended on a G protein-dependent signaling cascade because current inhibition was lost in the presence of GDP- β -S, PTX, or pp60c-src tyrosine kinase inhibitory peptide (Callaghan et al., 2008). From this unusual profile, the group screened a variety of receptors expressed in DRG neurons that might mediate Vc1.1's inhibitory actions on $\text{Ca}_v2.2$ activity. Surprisingly, only GABA_B receptor antagonists occluded N-current inhibition by Vc1.1 and Rg1A (Callaghan et al., 2008; Callaghan and Adams, 2010). Concomitantly, the GABA_B receptor agonist baclofen and Vc1.1 were nonadditive in their inhibitory effects. Vc1.1 and Rg1A inhibited N current in mouse DRG neurons from $\alpha 9$ nicotinic receptor subunit knockout mice, confirming that Vc1.1's actions occurred independently of nicotinic receptors because $\alpha 10$ is unable to form functional channels without the $\alpha 9$ subunit. A GABA_B receptor antagonist blocked toxin-mediated relief from allodynic pain in animal studies (Klimis et al., 2011), consistent with Vc1.1 somehow acting via GABA_B receptors and independently of nicotinic receptor subunits.

In most DRG neurons, GABA_B receptors inhibit N current by a $\text{G}\beta\gamma$ -mediated, voltage-dependent mechanism as noted above. However, in a subpopulation of DRG neurons, baclofen modulates N current by an

additional PTX-sensitive, $G\beta\gamma$ -independent and voltage-independent mechanism involving c-src kinase (Diversé-Pierluissi et al., 1997; Raingo et al., 2007). The Lipscombe laboratory and their colleagues previously identified a $Ca_v2.2$ splice variant selectively enriched in capsaicin-responsive DRG nociceptive neurons that exhibits both voltage-dependent and independent inhibition of N current by baclofen (Bell et al., 2004; Raingo et al., 2007). In exon [37a] of $Ca_v2.2$, a 14-amino acid insert in the proximal end of the carboxy-terminal tail contains two tyrosine residues (Y1743 and Y1747), whereas a phenylalanine has replaced the second tyrosine in the mutually exclusive exon [37b]. Voltage-independent inhibition of $Ca_v2.2e$ [37a] requires phosphorylation of the second but not the first tyrosine by c-src kinase. If mutated to phenylalanine (Y1747F) as is found in $Ca_v2.2e$ [37b], voltage-independent inhibition is lost and only voltage-dependent inhibition remains (Raingo et al., 2007). In contrast, complete relief of $Ca_v2.2e$ [37b] current inhibition by baclofen occurs with positive voltage, indicating that the 37b splice variant is insensitive to voltage-independent inhibition by c-src kinase. Collectively, the findings suggest that as with baclofen, $Vc1.1$ binds to $GABA_B$ receptors activating the same voltage-independent signaling pathway in DRG neurons to selectively inhibit $Ca_v2.2e$ [37a]. Whether other Ca_v2 family members could be modulated by this pathway remained an open question.

Here, Berecki et al. (2014) tested human recombinant $Ca_v2.1$ and $Ca_v2.3$ channels expressed in HEK 293T cells along with the $GABA_B$ receptor to answer the question of whether other Ca_v2 family members are sensitive to baclofen and/or $Vc1.1$ modulation by $GABA_B$ receptors (Fig. 1 A). Although the traditional $GABA_B$ receptor agonist baclofen inhibited currents from both channels, $Vc1.1$ and its cyclized form, $cVc1.1$, inhibited $Ca_v2.3$ but not $Ca_v2.1$ currents. The $GABA_B$ receptor antagonist CGP55845 had no effect on currents itself but antagonized the actions of $cVc1.1$, indicating that it too must somehow activate the $GABA_B$ receptor. The characteristics of $Ca_v2.1$ modulation by baclofen fit classical $G\beta\gamma$ -mediated, voltage-dependent inhibition (Fig. 1 B), as prepulses relieved all the inhibition (Diversé-Pierluissi et al., 1997). In contrast, $Ca_v2.3$ current inhibition by either baclofen or $cVc1.1$ shows no voltage-dependent modulation; prepulses were unable to relieve any of the inhibition. As with $Ca_v2.2$, inhibition requires c-src kinase because coexpression with a double mutant, inactive src kinase or synthetic pp60c-src kinase inhibitory peptide blocked $Ca_v2.3$ current inhibition, whereas the opposite treatment, overexpression of wild-type c-src kinase, enhanced inhibition (Fig. 1 C).

Sequence analysis revealed that $Ca_v2.3$ has two tyrosines (Y1761 and Y1765) in its C-terminal tail homologous to the two tyrosines in $Ca_v2.2e$ [37a]'s proximal C terminus (Raingo et al., 2007). $Ca_v2.3$ mutagenesis

revealed that the first (Y1761) but not the second (Y1765) tyrosine is required for voltage-independent current inhibition, presumably because its phosphorylation is required. In contrast, the critical tyrosine-containing amino acid motif found in the C termini of both $Ca_v2.2e$ [37a] and $Ca_v2.3$ is absent in $Ca_v2.1$, which may explain this channel's insensitivity to voltage-independent modulation. To test that possibility, a tyrosine residue was swapped in to either of the two homologous sites in the C terminus of $Ca_v2.1$, and current was retested for sensitivity to $cVc1.1$. A small amount of current inhibition was now

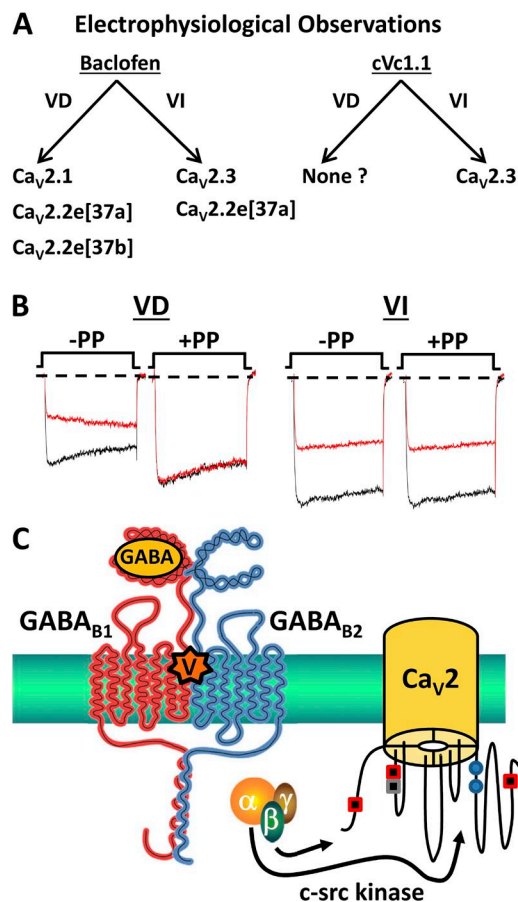


Figure 1. $GABA_B$ receptor activation elicits both voltage-dependent and voltage-independent inhibition of Ca_v2 current. (A) After $GABA_B$ receptor activation, Ca_v2 family members exhibit distinct modulation by voltage-dependent (VD) and/or voltage-independent (VI) current inhibition. (B) Example current traces illustrate the two forms of modulation. (Left traces) VD inhibition exhibits slowed kinetics (red traces) compared with control (black) currents that is relieved with positive prepulses (PP). (Right traces) VI inhibition (VI) remains following a PP. (C) Schematic of the proposed mechanism for Ca_v2 inhibition by the two pathways. $GABA_{B1}$ and $GABA_{B2}$ subunits form the $GABA_B$ receptor. $GABA$ or baclofen binds to the B1 subunit, activating the receptor. $cVc1.1$ (V) binds at an undefined site within the interface of the two subunits. Activated $G\beta\gamma$ binds to multiple sites (red squares), whereas $G\alpha$ stimulates c-src kinase to phosphorylate tyrosine residues (blue circles) in the C-terminal tail of certain Ca_v2 family members. Black squares, $Ca_v\beta$ high affinity-binding sites.

observed with each point mutation, indicating that tyrosine phosphorylation in the proximal C-terminal tail is critical for c-src kinase modulation of channels.

These and previous findings from Adams and colleagues (Callaghan et al., 2008; Callaghan and Adams, 2010; Clark et al., 2010; Cuny et al., 2012) associate three toxins, Rg1A, Vc1.1, and cVc1.1, with GABA_B receptor activation; however, whether they bind directly to the receptor or to another target protein remains controversial (McIntosh et al., 2009). One B1 and one B2 subunit heterodimerize to form a functional GABA_B receptor (White and Cousins, 1998). B1 binds GABA in its large extracellular N-terminal domain. B2 is required for high affinity agonist binding to B1, contains a binding site for allosteric modulators, and recruits the G protein (Benke, 2013). Studies using siRNA to lower either B1 or B2 levels found that baclofen, Vc1.1, and Rg1A require both B1 and B2 subunits for N-current inhibition in DRG neurons, consistent with binding to GABA_B heterodimers (Cuny et al., 2012). When Berecki et al. (2014) transfected HEK 293T cells with only the B1 subunit of the GABA_B receptor, Ca_v2.3 inhibition by both baclofen and cVc1.1 was lost, supporting—but not proving—the notion that the toxin is somehow interacting with the receptor to elicit its activation. Potentially confounding this notion are findings that very high concentrations of Vc1.1 cannot not displace ³H-CGP-5462 binding from human recombinant B1b/B2 GABA_B receptors (McIntosh et al., 2009) or from binding in DRG neurons (Adams and Berecki, 2013). These findings indicate that Vc1.1 does not compete with baclofen for a binding site but raise several questions surrounding Vc1.1's site of action. In particular, if Vc1.1 does bind to the GABA_B receptor, where is the binding site located, and how does toxin binding activate the receptor? Recently, molecular dynamic simulation studies of toxin binding to GABA_B receptors identified a putative toxin-binding site at the internal B1/B2 heterodimerization interface (Adams and Berecki, 2013). Whether toxins do indeed bind an ectodomain to activate GABA_B receptors awaits further binding, structure–function, and crystal structure studies. Once the toxin-binding site is confirmed, functional questions can be addressed, including whether other splice variants of Ca_v2.3, which contain the critical tyrosine residue in the C terminus tail, are similarly modulated, and where in the pain pathway are Ca_v2.3 and GABA_B receptors found within the same macromolecular signaling complex.

This insightful study by Berecki et al. (2014) demonstrates how the elaborate details of signaling by individual G protein–coupled receptors (GPCRs), in this case GABA_B receptors, can give rise to a remarkable level of control over specific members of the Ca_v2 family. By characterizing the details of each branch of GABA_B receptor signaling, potential therapeutic targets can be identified to strategically treat specific types of pain.

Indeed, behavioral studies using siRNAs for Ca_v2.2e[37a] indicate that this channel selectively participates in mediating normal thermal nociception as well as thermal hyperalgesia that accompanies nerve injury (Altier et al., 2007; Andrade et al., 2010). Splice variants of Ca_v2 channels exhibit different voltage and gating properties that will affect transmitter release and membrane excitability (Bell et al., 2004). Furthermore, whether a channel can be modulated will depend on its splicing and on the colocalization of critical signaling molecules such as c-src kinase within a sensory neuron. Lastly, expression of a particular GPCR within any given sensory neuron will determine whether modulation could be initiated by a transmitter such as GABA or a ligand such as cVc1.1. In this study, rather than using a toxin to directly inhibit ion channel activity, a toxin was used to activate a GPCR to inhibit the activity of a subset of Ca_v2 channels. Moreover, cVc1.1 is ~1,000 times more potent than baclofen in inhibiting Ca_v2.3 currents. Most exciting is previous findings that oral delivery of cVc1.1 produces significant pain relief in the chronic constriction injury rat model of neuropathic pain (Clark et al., 2010). Whether cVc1.1 will find its way into the pharmacological toolbox of treatments for clinical neuropathic pain awaits future studies. Who knew that such a simple question of whether GABA modulates Ca_v2.3 currents would yield a new target, ligand, and potential mechanism for treating neuropathic pain?

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