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R e v i e w

Structure, function and regulation of $Ca_v2.2$ N-type calcium channels

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Abstract. N-type or $Ca_V2.2$ high-voltage activated calcium channels are distinguished by exclusively neuronal tissue distribution, sensitivity to ω-conotoxins, prominent inhibition by G-proteins, and a unique role in nociception. Most investigated modulatory pathway regulating the $Ca_V2.2$ channels is G-protein-coupled receptor-activated pathway leading to current inhibition by Gβγ subunit of G-protein. Binding of Gβγ dimer to α1 subunit of the $Ca_V2.2$ channel transfers the channel form "willing" to "reluctant" gating state. Channel phosphorylation by protein kinase C potentiates N-type calcium current. $Ca_V2.2$ channels could be functionally regulated also by a number of protein-protein interactions. $Ca_V2.2$ null mice are hyposensitive to inflammatory and neuropathic pain, otherwise they have a mild phenotype. Consistent with the mild phenotype of the $Ca_V2.2^{-/-}$ mice, reports on mutations linked to a disease phenotype are scarce. Only one mutation related to human heritable diseases was identified until now. Pharmaceutical inhibition of $Ca_V2.2$

channels either by direct inhibition of the channel, by an activation of G-protein coupled receptors, or by inhibition of membrane targeting of the channel protein are promising strategies for treatment of severe chronic and/or neuropathic pain.

Key words: N-type calcium current — $Ca_V2.2$ channel — auxiliary subunits — G-protein regulation — $G_{\beta\gamma}$ subunit — channel gating — channelopathies — inflammatory pain — neuropathic pain — proteomic analysis

Introduction

N-type or $Ca_V2.2$ calcium channels belong to the family of high-voltage activated calcium channels. Nowycki, Fox, and Tsien (Nowycky et al. 1985) were the first to suggest existence of three types of Ca channel in sensory neurones of the chick dorsal root ganglion. In addition to already known L-type and T-type currents they identified a third type of conductance – "N", that was neither T nor L. N-type calcium current was distinguished from an L-type by more negative voltage threshold for current inactivation and insensitivity to dihydropyridines, from a T-type it differed in more positive voltage threshold for activation. Soon thereafter, Wanke and coauthors (Wanke et al. 1987) demonstrated that N-type channels in superior cervical ganglions of rat are regulated by muscarinic pathway *via* pertussis toxin-sensitive G-proteins. Gross and Macdonald (1987) suggested modulation of N-type calcium current in mouse dorsal root ganglion (DRG) neurons by opioid receptors-related pathway. As a pharmacological tool for isolation of N-type calcium channels ω-conotoxins were established (Reynolds et al. 1986; Kasai et al. 1987).

Cloning of genes for individual calcium channel proteins during 1990-ties enabled extensive studies of their structure, regulation, and tissue distribution. Also, it formed a basis for new systemic nomenclature of voltage-gated ion channels. N-type calcium channel was named $Ca_V2.2$ in this nomenclature (Ertel et al. 2000).

Unique role of N-type in nociception was established (Malmberg and Yaksh 1994b; Omote et al. 1996). Additionally, dysregulation of these channels in fragile X mental retardation (Ferron et al. 2014) was reported and gain-of-function mutation

associated with myoclonus-dystonia syndrome was identified in one family (Groen et al. 2015).

Cloning of the $Ca_V2.2$ α1 subunit

In early 1990-ties, several cDNAs clones encoding α_1 subunits homologous to α_1 heart and skeletal subunit were isolated and characterized from rat brain (Snutch et al. 1990). Next, 240 kDa protein of N-type channel was detected by photolabeling and its association with $\alpha_2\delta$ subunit was evidenced (Ahlijanian et al. 1991). One year later, full-length sequence of 2336 amino acid protein of α_{1B} subunit in rat brain was described and immunoprecipitation labeling experiments restricted its expression to nervous system of the rat forebrain (Dubel et al. 1992). Westenbroek and coauthors recognized in rat brain two α_{1B} proteins with sizes of 240 and 210 kDa localized complementary in dendrites, nerve terminals and cell bodies of most neurons (Westenbroek et al. 1992).

Immunoprecipitation experiments showed presence of at least two different forms of α_1 subunits forming N-type calcium channel complex (Westenbroek et al. 1992). Human neuronal α_1 subunit designated as α_{1B} was identified in human neuroblastoma cells and central nervous system (Williams et al. 1992a, 1992b). Consistent with cloning of two isoforms of rat N-type calcium channel, two isoforms of human calcium channel α_{1B} subunit differing in their C-terminus were identified: the α_{1B}-1 comprising 2339 amino acids with the molecular weight of 262 kDa and the α_{1B}-2 with 2237 amino acids with the calculated molecular weight of 251 kDa.

Auxiliary subunits of the $Ca_V2.2$ channel complex

Expression of several subtypes of the auxiliary subunits gives rise to functionally different heterologous N-type channel complexes (McEnery et al. 1991; Williams et al. 1992b; Witcher et al. 1993; Gao et al. 2000). cDNA cloning analysis revealed expression of β subunit with high homology to the β subunit of rabbit skeletal DHP-sensitive calcium channel in the rat brain (Pragnell et al. 1991). Following studies identified three different β subunits, β1b, β3 and β4, which associate with the α_{1B} subunit of N-type

calcium channels and account for the diverse channel kinetic properties in different types of neurons (Scott et al. 1996; Witcher et al. 1993).

Generally, expression of principal α_{1B} subunit in complex with a β subunit enhances current throught the channel complex by increasing channel expression in plasma membrane and its opening probability. β subunit also modulates channel gating by shifting the voltage dependence of its activation and inactivation (Buraei and Yang 2010; Dolphin 2012). Biophysical data has shown that expression of the α_{1B} subunit of N-type calcium channel together with auxiliary β2 and $\alpha_2\delta$ subunits in HEK 293 cells resulted in barium current with properties typical for N-type current (Williams et al. 1992b). Experiments expressing α_{1B} subunit together with β1b revealed the increase in the whole-cell current and in rate of activation accompanied by the shift of the voltage-dependence of the inactivation to the hyperpolarized potentials. Results of this study did not show significant effect of co-expression of the α2δ subunit (Stea et al. 1993). Following study has described decelerated current inactivation, shift of the current-voltage relationship to negative potentials and modified voltage-dependent inactivation when β1b subunit was expressed in the complex with α_{1B}. In this study, co-expression of an α2δ subunit also affected the current properties by accelerating of both current activation and inactivation and by the shifting the I-V slightly to the more positive membrane potentials (Wakamori et al. 1999). In contrast, β3 demonstrated negative regulatory effect on N-type calcium channel complex suppressing current amplitude under physiological conditions and by shifting voltage-dependent inactivation to negative potentials (Yasuda et al. 2004). In contrast co-expression of the β4 subunit in N-type calcium channel complex resulted in increase in current density, a shift of activation to negative potentials, and deceleration of voltage-dependent inactivation (Canti et al. 2000; Stephens et al. 2000). Co-expression of α2δ subunit increases current amplitude by increasing an expression of the N-type channel complex in plasma membrane and addition of β3 subunit further stimulates membrane trafficking of the channel (Brust et al. 1993; Canti et al. 2000). Functional activity of N-type channel was increased by co-expression of β3 or β1b subunit, respectively, together with either α2δ1 or α2δ2 subunit in Xenopus oocytes, showing 9-fold peak current amplitude raise (Canti and Dolphin 2003; Gao et al. 2000). N-type channel complex with the α2δ1 but not the α2δ2 subunit

exhibited enhanced channel inactivation (Gao et al. 2000). α2δ1 and α2δ2 subunits had little effect on kinetics of N-type calcium current activation but accelerated current inactivation when coexpressed with the β1b subunit (Canti and Dolphin 2003).

Quantitative proteomic analysis showed that β4 followed by β3 are most frequently assembled with $Ca_V2.2$ α_1 subunit while β1 is less frequently and β2 is rarely forming a part of $Ca_V2.2$ channel complex (Muller et al. 2010). Recently, it was shown that the auxiliary $\alpha_2\delta$-1 subunit is necessary for membrane trafficking of α_{1B} subunit in dorsal root ganglion neurons and important for trafficking in dorsal horn neurons (Nieto-Rostro et al. 2018).

Role of $Ca_V2.2$ channels in physiology and pathophysiology

Expression of $Ca_V2.2$ channels was identified almost exclusively in central and peripheral neurons (Nowycky et al. 1985). These channels are located mainly in dendritic shafts and presynaptic terminals (Westenbroek et al. 1992). They were found in laminae I and II of the dorsal horn and were shown to be predominant at synapses that carry nociceptive information into the spinal cord (Westenbroek et al. 1998). Within II-III domain linker of the $Ca_V2.2$ α_1 subunit a synaptic protein interaction (synprint) site is located. This site interacts with proteins of the synaptic vesicle release complex, such as syntaxin 1 and SNAP-25 (Sheng et al. 1994, 1996). This interaction stabilizes membrane localization of $Ca_V2.2$ channels in close proximity to synaptic vesicles. Incoming action potentials activate large temporally precise calcium influx through $Ca_V2.2$ channels and activate release of neurotransmitters like glutamate or substance P (Weber et al. 2010) ensuring transmission of peripheral signals to the brain (Figure 1). This pathway conveys various sensory modalities from peripheral sensory neurons including pain, itch, touch, and perception of body muscle tension (Bourinet et al. 2014). In accord with suggested role of $Ca_V2.2$ channels in nociception, their inhibition suppressed formaline-induced pain (Malmberg and Yaksh 1994a, 1994b), thermally- and mechanically-induced pain (Omote et al. 1996), pain elicited by chemical irritants, and inflammatory pain (Vanegas and Schaible 2000). Mice constitutively lacking the $Ca_V2.2$ channels were hyposensitive to inflammatory and neuropathic pain (Hatakeyama et al. 2001; Kim et al. 2001; Saegusa et

al. 2001). Generally, $Ca_V2.2$ null mice have a mild phenotype that includes deficits for sympathetic control of heart rate and blood pressure (Mori et al. 2002), hyperactivity (Beuckmann et al. 2003), and reduced voluntary ethanol intake and reduced hypnotic response to alcohol (Newton et al. 2004). Because of their exclusive role in nociception and relatively minor contribution to other physiological processes $Ca_V2.2$ channels are attractive therapeutic target for analgesics.

Regulation of $Ca_V2.2$ channels by G-proteins

Regulation by G-proteins is perhaps the most investigated pathway modulating activity of the $Ca_V2.2$ channels. G-proteins are heterotrimers consisting of three subunits – α, ß, and γ. These intracellular complexes are bind to plasma membrane G-protein-coupled receptors (GPCR) (Duc et al. 2015). Activation of GPCR by agonist results in the dissociation of the G-protein heterotrimer into Gα and Gβγ subunits. The separated subunits interact with various effectors molecules including an α_1 subunit of voltage gated calcium channels (Dolphin 1995). Most prominent current modulation by a Gβγ dimer was described for the $Ca_V2.2$ channel (Zhang et al. 1996; Currie and Fox 1997). Gβγ dimer binds directly to the α_1 subunit of the $Ca_V2.2$ channels and initiates a transition of the channel from "willing" into "reluctant" gating state (Bean 1989). Reluctant gating state is characterized by a slow activation kinetics, shift of voltage dependence of channel activation towards more positive membrane voltages, and lower whole cell current amplitude (Bean 1989; Hille 1994; Carabelli et al. 1996; Colecraft et al. 2000). Current inhibition caused by Gβγ binding can be relieved by a brief depolarization to positive membrane voltages – so-called prepulse facilitation (Elmslie et al. 1990; Zhang et al. 1996; Currie and Fox 1997; Herlitze et al. 2001). Such mechanism of regulation of calcium entry *via* $Ca_V2.2$ channels enables modulation of neurotransmitter release and consequently modulation of the synaptic transmission of nociceptive signal by activation of synaptic GPRCs.

Reluctant gating state is characterized by a direct binding of the Gβγ dimer to the $Ca_V2.2$ α_1 subunit. Multiple interaction sites were identified in the $Ca_V2.2$ α_1 sequence (Figure 2): the aminoterminal part (Page et al. 1998; Stephens et al. 1998; Agler et al.

2005; Page et al. 2010), the intracellular loop connecting domains I and II (De Waard et al. 1997; Herlitze et al. 1997; Page et al. 1997; Simen and Miller 1998; Van Petegem et al. 2004; Tedford et al. 2010), and the carboxyterminal part (Zhang et al. 1996; Qin et al. 1997).

In addition to a direct protein-protein interaction, activation of membrane-located GPRCs in cells expressing $Ca_V2.2$ channels stimulates additional modulatory pathways involving intracellular signaling messengers like PIP_2 (Keum et al. 2014) and/or arachidonic acid (Mitra-Ganguli et al. 2009). PIP_2-dependent pathway inhibits N-type calcium current by a voltage-independent, Ca_V β subunit isoform-dependent mechanism (Vivas et al. 2013; Keum et al. 2014).

Regulation of $Ca_V2.2$ channel by protein kinases

$Ca_V2.2$ channels are phosphorylated by protein kinase C (PKC) isozymes PKCß (Constantin et al. 2017), PKCßII and PKCε (Rajagopal et al. 2009), and PKCδ (Rajagopal et al. 2011). This phosphorylation results in a potentiation of N-type calcium current which can be counteracted by a presence of an auxiliary ß subunit (Garcia-Ferreiro et al. 2001; Rajagopal et al. 2014). $Ca_V2.2$ channel is also constitutively phosphorylated by the calcium/calmodulin-dependent protein kinase II in resting sensory neurons and removal of this phosphorylation result in a loss of channel activity (Kostic et al. 2014).

Protein complexes formed by $Ca_V2.2$ channels

$Ca_V2.2$ channels could be functionally regulated by a number of protein-protein interactions. Proteomic analysis focused on identification of such interacting protein networks is fast growing omic research field. Most investigated interaction partners of $Ca_V2.2$ channel protein are G-proteins. Other interacting proteins were identified, as well. Activity of $Ca_V2.2$ is regulated by the binding of collapsin response mediator protein 2 (CRMP-2) to the channel domain I-II intracellular loop and the distal C terminus and enhances calcium current amplitude without altering its biophysical properties (Brittain et

al. 2009; Chi et al. 2009). This interaction requires phosphorylation of CRMP-2 by cyclin-dependent kinase 5 (Brittain et al. 2012; Kim and Ryan 2013). SNARE (soluble NSF attachment receptor) proteins form signaling complex with $Ca_V2.2$ channels in presynaptic membrane and mediate exocytosis (Catterall and Few 2008). MAP6 proteins, originally identified as microtubule stabilizing agents, bind to the $Ca_V2.2$ channel carboxyterminus and this interaction is necessary for proper membrane targeting of the channel protein (Brocard et al. 2017). Coimmunoprecipitation experiments demonstrated that D1 (Kisilevsky et al. 2008) and D2 (Kisilevsky and Zamponi 2008) dopamine receptors form a signaling complex with the $Ca_V2.2$ channels. In addition to G-protein-dependent channel modulation, both receptors affect membrane expression level of $Ca_V2.2$ channel protein.

More recently, high-throughput assays were used to identify potential $Ca_V2.2$-interacting proteins. Screen of presynaptic $Ca_V2.2$ complex members by an antibody-mediated capture of the channel from purified rat brain synaptosome lysate followed by mass spectroscopy identified 144 potentially interacting proteins (Khanna et al. 2007c). Within the presynaptic transmitter release site (TRS) the $Ca_V2.2$ channel associated with Munc18, spectrin, N-ethylmaleimide-sensitive factor (NSF), vesicle-associated membrane protein, and α-catenin (Khanna et al. 2007a). Analysis of TRS-associated endocytosis protein complex revealed specific binding between $Ca_V2.2$ channel and H-clathrin, L-clathrin, dynamin, and the adaptor proteins AP180 and intersectin (Khanna et al. 2007b).

Yeast-two hybrid screen identified a direct interaction of the central PDZ domain of RIM protein with carboxyterminus of the $Ca_V2.2$ channel (Kaeser et al. 2011). This binding is essential for channel tethering into presynaptic terminal. Another group used the yeast split-ubiquitin system to define the interactome of the $Ca_V2.2$ Ca^{2+} channel α_1 subunit and identified tetraspanin-13 (TSPAN-13) (Mallmann et al. 2013), reticulon 1 (RTN1), member 1 of solute carrier family 38 (SLC38), prostaglandin D2 synthase (PTGDS), and transmembrane protein 223 (TMEM223) (Mallmann et al. 2019) as interaction partners of the channel. Detailed electrophysiological studies revealed that TSPAN-13, TMEM223, and, to a lesser extent, PTGDS and SLC38, negatively

modulated Ca^{2+} entry required for transmitter release and/or for dendritic plasticity under physiological conditions. RTN1 modulated $Ca_V2.2$ channels only to a minor extent.

$Ca_V2.2$ channel-related channelopathies

Knockout of individual genes in animal models was established as usefull tool for gaining insight into the role of specific proteins in (patho)physiology. Mice with constitutive deletion of the $Ca_V2.2$ channel are viable and display relatively mild phenotype including hyperactivity (Beuckmann et al. 2003), reduced anxiety (Saegusa et al. 2001), a reduction of voluntary alcohol intake (Newton et al. 2004), and problems with blood pressure control (Mori et al. 2002). Reduced sensitivity to pain in $Ca_V2.2^{-/-}$ mice confirmed this channel as a prominent target in pain management (Hatakeyama et al. 2001; Kim et al. 2001; Saegusa et al. 2001).

Consistent with the mild phenotype of the $Ca_V2.2^{-/-}$ mice, reports on mutations linked to a disease phenotype are scarce. Congenital missense mutation resulting in R1389H amino acid exchange associated with myoclonus-dystonia (M-D) syndrome was identified in one family (Groen et al. 2015). When mutated channels were expressed, they conducted lower single-channel current but enhanced whole-cell current. Apparent gain-of-function phenotype can be explained by more frequent openings of mutated channel (Groen et al. 2015) and/or by decreased G-protein-dependent channel inhibition due to longer channel open time (Weiss 2015). Enhanced channel conductance is consistent with symptoms of the disease which include quick, involuntary muscle jerking or twitching (myoclonus). However, other group (Mencacci et al. 2015) contested this work. In their screening of a large cohort of 520 individuals with both familial an sporadic M-D syndrome they detected R1389H mutation in a single sporadic case with M-D, but in none of the 146 probands with familial M-D. No other mutations related to human heritable diseases were identified until now.

Blockers of N-type calcium channels in treatment of pain

Considering their specific involvement in nociception and lack of exclusive role in other physiological processes inhibition of $Ca_V2.2$ channels is a promising strategy for treatment of severe chronic pain. Four possible scenarios are available: i) direct inhibition of the channel; ii) inhibition of N-type calcium current by activation of GPCR; iii) inhibition of membrane targeting of the channel protein; iv) disruption of signaling protein complex (Figure 1).

Most known direct $Ca_V2.2$ channel blockers are toxins isolated from fish-hunting mollusks. Omega-conotoxin GVIA was identified as a very first specific blocker of the N-type calcium current (Olivera et al. 1984; Reynolds et al. 1986). Currently, number of other conotoxins acting as selective $Ca_V2.2$ channel blockers is known (Zamponi et al. 2015). Three novel ω-conotoxins from Conus catus were effective in treatment of inflammatory pain in mice (Sadeghi et al. 2013). In 2005, first FDA (Food and Drug Administration) approval was granted for clinical use of 2005 to ω-conotoxin MVIIA (Prialt, Ziconotide) (Schroeder et al. 2006). However, conotoxin do not cross the blood-brain barrier and must be applied intrathecally. Ziconotide still remains the only clinically used $Ca_V2.2$ channel blocker. Recently, series of pyrazolyltetrahydropyran $Ca_V2.2$ channel blockers was synthesized and tested for potential use in clinics. Two of these compounds did block N-type calcium current in patch clamp assay, were orally bioavailable in rats, and were efficacious in suppressing inflammatory and/or neuropathic pain (Wall et al. 2018). In addition to inhibition of L-type calcium current, some dihydropyridines also block N-type calcium current with high affinity. L/N-type channel blocker cilnidipine lowered post-dialysis systolic blood pressure in a small group of tested patients undergoing hemodialysis (Ito et al. 2019). Intradialytic hypertension is a common complication in such patients. It may be associated with sympathetic overactivity, therefore concurrent inhibition of L-type and N-type calcium current may represent a potential treatment.

Activation of GPRCs results in inhibition of the N-type calcium current by a dissociated Gβγ dimer. Most common clinically used analgesics acting *via* GPRC is μ-opoid receptor agonist morphine, however, its use is associated with severe side effects including addiction. Agonists of other opioid receptors, i.e., κ, δ, and nociceptin, also inhibit N-type calcium current. GPRCs are also coupled to other effectors including G-

protein-coupled potassium channels, therefore mechanism of therapeutic action of their ligands is complex and exceeds modulation of $Ca_V2.2$ channels (Zamponi et al. 2015).

Trafficking of $Ca_V2.2$ channel protein into plasma membrane depends on its interaction with CRMP-2 (Chi et al. 2009). This interaction can be disrupted by small interfering peptides. It was shown that suppression of CRMP-2 binding to the $Ca_V2.2$ channel complex reduces inflammatory and neuropathic pain (Brittain et al. 2011; Ripsch et al. 2012; Wilson et al. 2012). More detailed knowledge of complex protein-protein interactions in which $Ca_V2.2$ channels are involved may open new possibilities for specific suppression of their activation by agents penetrable through the blood-brain barrier allowing better therapeutic approach to intractable pain.

Conclusion

Altogether, $Ca_V2.2$ channels play an unique role in perception of pain. Having minor role in other physiological processes, they represent an attractive target for treatment of severe inflammatory and/or neuropathic pain. As use of blockers acting directly on channel protein is obstructed by their inability to cross blood-brain barrier, knowledge of complex signaling networks regulating $Ca_V2.2$ channels may be a promising way for new drug design.

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Figure legends.

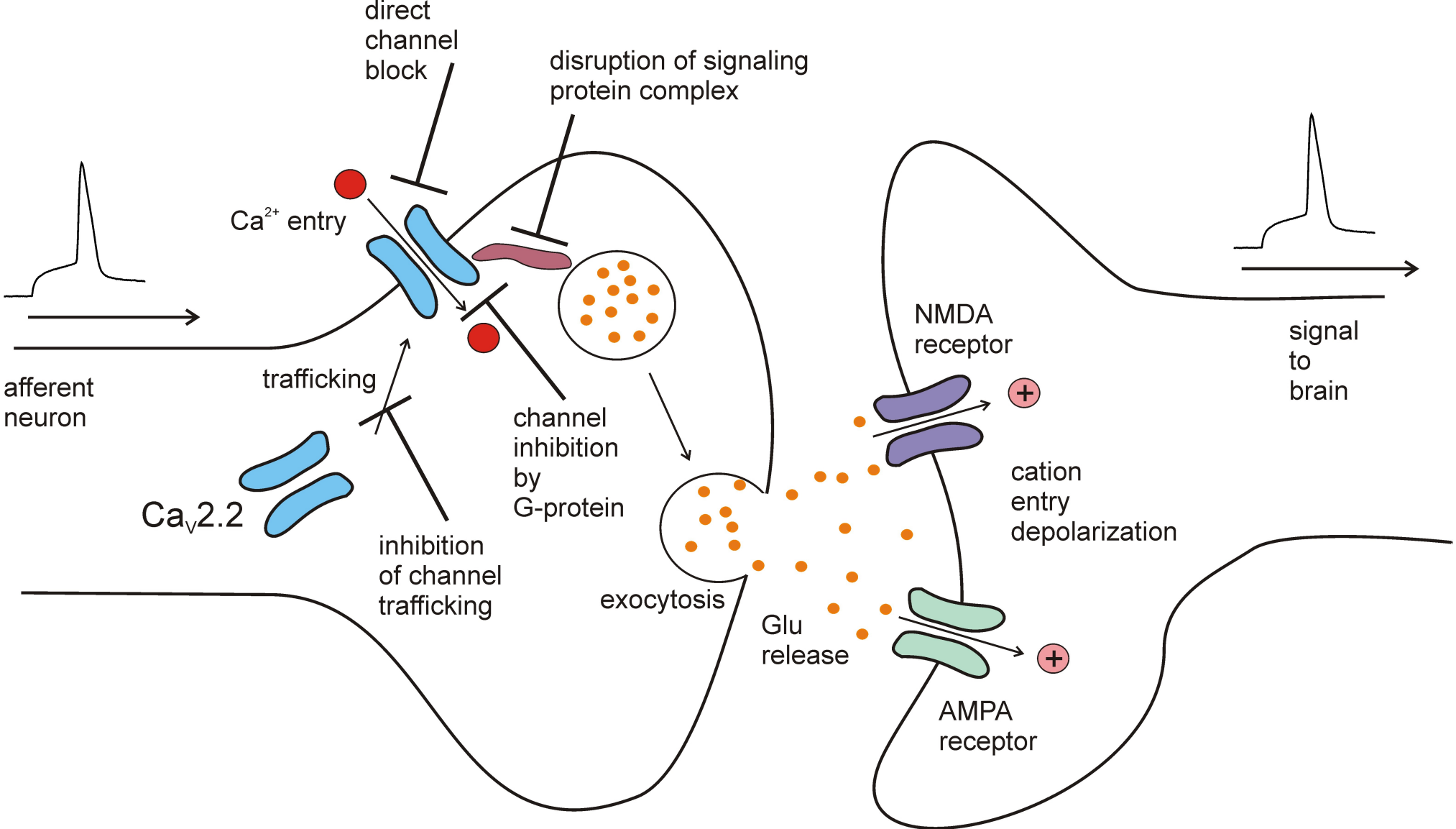
Figure 1. Role of $Ca_V2.2$ channels in synaptic transmission. $Ca_V2.2$ channels are expressed in presynaptic membrane of afferent nociceptive neurons. They form signaling complex with synaptic vesicles releasing glutamate. Depolarization of presynaptic membrane by incoming action potential results in activation of $Ca_V2.2$ channels. Calcium entry through these channels activates exocytosis and glutamate release. Glutamare (Glu) activates corresponding receptors in postsynaptic membrane of dorsal horn neuron. Cations entering through NMDA and AMPA receptors depolarize postsynaptic membrane and initiate signaling to the brain.

Figure 2. G-protein interaction sites. An outline of primary structure of the α_1 subunit of N-type calcium channel. Barrels (S1–S6) represent transmembrane segments in domains I–IV. Open boxes indicate regions in the α_1 subunit sequence interacting with the G-protein $G\beta\gamma$ subunit.

Table 1. Effects of co-expression of auxiliary subunits of the $Ca_V2.2$ channel complex on current through the α_{1B} subunit

	Effect on N-type current	Reference
ß subunit		
ß1b	↑ I_{Ca} amplitude, ↑I_{Ca} activation, negative shift of voltage-dependent inactivation	*Stea 1993*
	↓ I_{Ca} inactivation, negative *I-V* shift, negative shift of voltage-dependent inactivation	*Wakamori 1999*
	↑ I_{Ca} inactivation	*Canti 2003*
ß3	↓ I_{Ca} amplitude, negative shift of voltage-dependent inactivation	*Yasuda 2004*
	↑ I_{Ca} amplitude, ↑ channel expression	*Brust 1993, Canti 2003*
ß4	↑ I_{Ca} density, negative shift of I_{Ca} activation, ↓voltage-dependent inactivation	*Canti 2000, Stephens 2000*
α2δ subunit		
α2δ1	↔ I_{Ca} amplitude	*Stea 1993*
	↑ I_{Ca} activation, ↑ I_{Ca} inactivation, positive *I-V* shift	*Wakamori1999*
	↑ I_{Ca} amplitude, ↑ channel expression	*Brust1993, Canti 2003*
	↑ I_{Ca} amplitude, ↑ I_{Ca} inactivation	*Gao 2000*
	↔ I_{Ca} activation	*Canti 2003*
α2δ2	↑ I_{Ca} amplitude, ↔ I_{Ca} inactivation	*Gao 2000*
	↔ I_{Ca} activation	*Canti 2003*

Arrows denote effects of auxiliary subunits on biophysical properties of the $Ca_V2.2$ channel complex: ↑ increase/acceleration of the current attribute, ↓ decrease/slowing of the current attribute, ↔ no or little effect on current attribute.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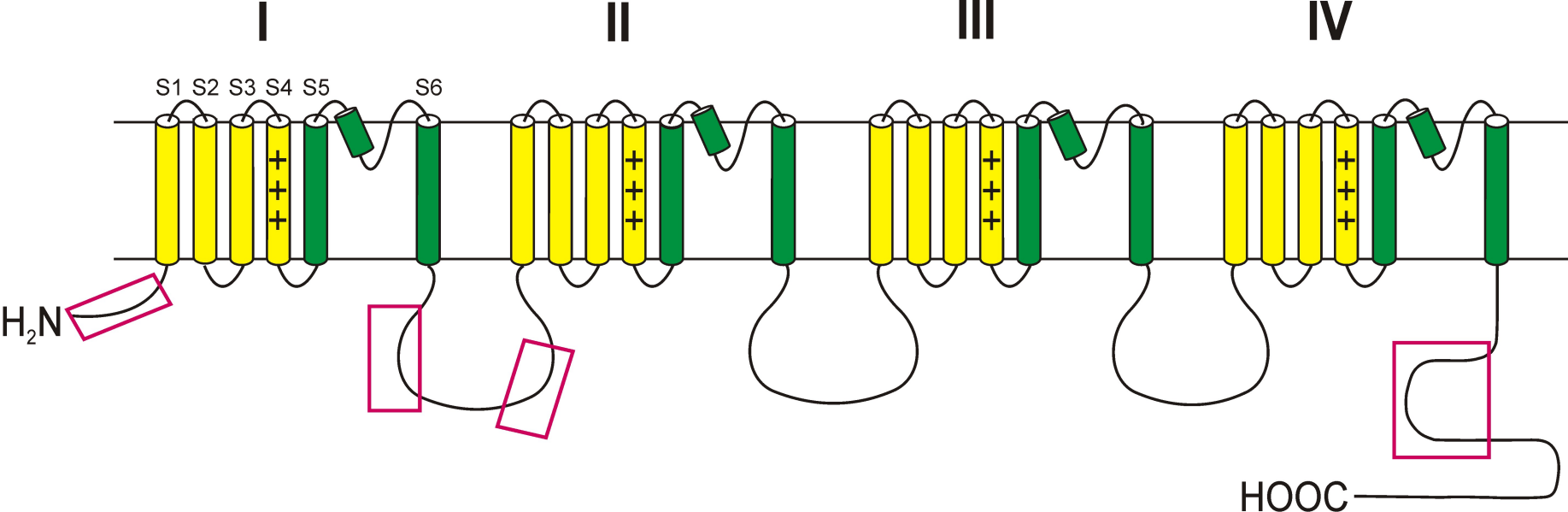
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