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Emerging evidence for specific neuronal functions of auxiliary calcium channel $\alpha_2\delta$ subunits

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Abstract

In nerve cells the ubiquitous second messenger calcium regulates a variety of vitally important functions including neurotransmitter release, gene regulation, and neuronal plasticity. The entry of calcium into cells is tightly regulated by voltage-gated calcium channels, which consist of a heteromultimeric complex of a pore forming α_1 , and the auxiliary β and $\alpha_2\delta$ subunits. Four genes (*Cacna2d1-4*) encode for the extracellular membrane-attached $\alpha_2\delta$ subunits ($\alpha_2\delta$ -1 to $\alpha_2\delta$ -4), out of which three isoforms ($\alpha_2\delta$ -1 to -3) are strongly expressed in the central nervous system. Over the years a wealth of studies has demonstrated the classical role of $\alpha_2\delta$ subunits in channel trafficking and calcium current modulation. Recent studies in specialized neuronal cell systems propose roles of $\alpha_2\delta$ subunits beyond the classical view and implicate $\alpha_2\delta$ subunits as important regulators of synapse formation. These findings are supported by the identification of novel human disease mutations associated with $\alpha_2\delta$ subunits and by the fact that $\alpha_2\delta$ subunits are the target of the anti-epileptic and anti-allodynic drugs gabapentin and pregabalin. Here we review the recently emerging evidence for specific as well as redundant neuronal roles of $\alpha_2\delta$ subunits and discuss the mechanisms for establishing and maintaining specificity.

Keywords

Voltage-gated Ca^{2+} channels; Presynaptic; Postsynaptic; Synaptogenesis; CNS

1. Introduction

In the central nervous system (CNS) calcium entering nerve cells through voltage-gated calcium channels (Ca_v s) mediates and regulates a variety of neuronal functions ranging from neurotransmitter secretion and postsynaptic signal integration to gene regulation and neuronal plasticity. For example, presynaptic Ca_v s trigger neurotransmitter release (Stanley, 1993) and postsynaptic Ca_v s are involved in the transcriptional regulation of CREB (cAMP-responsive element-binding protein) and NFAT (nuclear factor of activated T cells) (Deisseroth et al. 2003; Dolmetsch 2003) and thus modulate the excitability and likely play a

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crucial part in the formation of new memory (Moosmang et al. 2005). Over the recent years a detailed picture on the distribution and function of pre- and postsynaptic calcium channel types has begun to emerge (Obermair and Flucher 2013) and the importance of Ca_V s is emphasized by the existence of channelopathies caused by loss-of-function as well as gain-of-function mutations (Pietrobon 2010; Striessnig et al. 2010). Calcium channels are organized in heteromultimeric complexes consisting of the pore-forming α_1 subunit and the auxiliary β and $\alpha_2\delta$ subunits. The α_1 subunit defines the basic biophysical, pharmacological and physiological properties of the channels while the β and $\alpha_2\delta$ subunits are involved in the localization, trafficking and stabilization of the channel complex (reviewed in Arikath and Campbell 2003; Obermair et al. 2008; Dolphin 2009; Buraei and Yang 2010). The majority of today's knowledge on the role of the individual channel subunits is based on studies in heterologous expression systems such as *Xenopus laevis* oocytes or human embryonic kidney (HEK) cells. However, the recent development of powerful neuronal expression systems and the characterization of Ca_V knockout and mutant animal models have provided novel insights into the physiological importance of auxiliary β and $\alpha_2\delta$ subunits in neuronal functions such as the regulation of gene expression as well as synaptic function. While these studies provide evidence for specific roles of individual Ca_V subunits, they also indicate that some of their basic functions may be rather common and redundantly shared between subunit isoforms. Therefore the challenge of ongoing and future investigations is to elucidate whether and how auxiliary Ca_V subunit isoforms and their respective splice variants contribute to the various neuronal functions. This is not merely relevant for our understanding of neuronal signaling, but may open up new therapeutic strategies for treating CNS diseases involving Ca_V s such as neurodegenerative diseases, epilepsy, or anxiety and mood disorders. Here we review recent evidence for both specific and redundant functions of auxiliary Ca_V subunits with a focus on the extracellular $\alpha_2\delta$ subunits.

2. Neuronal calcium channel complexes

2.1. Subunit composition

The core complex of neuronal voltage-gated calcium channels consists of an α_1 subunit, which contains the ion-conducting pore, and the auxiliary β and $\alpha_2\delta$ subunits (Fig. 1A). In skeletal muscle also a γ subunit is part of the complex; however, in the nervous system various γ subunits act primarily as transmembrane AMPA receptor regulatory proteins (TARPs; Jackson and Nicoll 2011).

The α_1 subunit: The α_1 subunits of voltage-gated calcium channels are a family of 10 genes involving the high-voltage-activated classes Ca_V1 and Ca_V2 (α_{1A} to α_{1F} and α_{1S}), and the low-voltage-activated class Ca_V3 (α_{1G} to α_{1I}) (Catterall 2011). Each α_1 subunit consists of four homologous repeats (I to IV), each with six membrane spanning domains (S1 to S6). Specific functions have been assigned to particular structures within this sequence, like the voltage sensor in the four S4 segments and the ion selectivity filter of the conductance pore in the loops between S5 and S6 of each repeat. Depolarization of the neuronal plasma membrane leads to a conformational change within the α_1 subunit that gates the channel and activates the calcium current. Domains involved in protein-protein interaction with the accessory channel subunits, regulatory proteins, and other channels have

been localized in the large intracellular loops connecting the homologous repeats and in the long C-terminal tail. The α_1 subunit also defines the basic biophysical and pharmacological properties due to important drug binding sites such as dihydropyridines, phenylalkylamines, as well as benzothiazepines (Catterall 2011). α_1 subunit splicing increases the functional heterogeneity and the spectrum of pharmacological characteristics (Koschak 2010; Flucher and Tuluc 2011). Out of the four members of the dihydropyridine-sensitive L-type (Ca_V1) calcium channels, $\text{Ca}_V1.2$ and $\text{Ca}_V1.3$ are expressed in the CNS and play important roles in pace-making ($\text{Ca}_V1.3$), postsynaptic signal integration, and excitation-transcription coupling in neurons. L-type calcium channels have mainly been associated with postsynaptic functions with two notable exceptions. $\text{Ca}_V1.3$ and $\text{Ca}_V1.4$ regulate presynaptic glutamate release in two highly specialized synapses, namely auditory hair cells and retinal photoreceptor cells, respectively. The P/Q-, N-, and R-type calcium channels ($\text{Ca}_V2.1$, 2.2, and 2.3) can be specifically blocked with a number of invertebrate toxins (Catterall 2011), and their chief function is controlling neurotransmitter release in synapses (Nimmervoll et al. 2013). The members of the low-voltage activated, T-type calcium channels (Ca_V3 family) are highly expressed during early development of many cell types, can be blocked by Ni^{2+} , and contribute to the excitability and pace-making in neurons (reviewed in Perez-Reyes 2003).

The β subunit: The cytoplasmic β subunit consists of a conserved SH3 protein interaction domain and a nucleotide kinase-like domain (Chen et al. 2004; Opatowsky et al. 2004; Van Petegem et al. 2004) and thus resembles in structure the membrane-associated guanylate kinase proteins (Dolphin 2003; Takahashi et al. 2005). However, the SH3 domain of β subunits differs from that of canonical polyprolin-binding pockets and the guanylate kinase fold is modified so that it lacks kinase activity. Instead it binds the intracellular I-II linker of α_1 subunits at the so-called α -interaction-domain (AID) with nanomolar affinity (De Waard et al. 1995; Van Petegem et al. 2008). The SH3 and the GK-like domain are highly conserved among the four genes encoding β subunits (*Cacnb1-b4*). The sequences connecting these domains as well as the N- and C-termini vary between isoforms and are subject to alternative splicing (Colecraft et al. 2002; Dolphin 2003). In the channel complex β subunits serve two roles: They have a chaperon function regulating the export of the calcium channel from the endoplasmic reticulum and thus membrane expression of functional channels (Obermair et al. 2010; Fang and Colecraft 2011). Moreover, they modulate gating properties of the channel directly as well as by interaction with other regulatory proteins like Rab binding proteins or G-proteins. β itself is subject to PKA mediated phosphorylation (reviewed in Buraei and Yang 2010). The β_{2a} isoform is palmitoylated at two N-terminal cysteines and therefore membrane-associated even in the absence of an α_1 subunit. Nevertheless, the association of β subunits with the channel complex entirely depends on their binding to the AID in the α_1 subunit. This binding site in the cytoplasmic loop between repeats I and II of the α_1 subunit is a unique feature of the Ca_V1 and Ca_V2 subclasses of Ca_V s. Accordingly, at least in heterologous expression systems all β subunits can associate with any of the Ca_V1 or Ca_V2 members but not with the low-voltage-activated calcium channels of the Ca_V3 subclass (Dolphin 2003). Because of their central role in regulating functional expression and biophysical properties of calcium channels, and because of the well-defined interaction site (see above), interference with the

AID- β interaction is an attractive strategy for designing specific calcium channel antagonists. Indeed, a competing AID peptide induced functional uncoupling of $\text{Ca}_V1.2$ and β_{2a} subunits (Hohaus et al. 2000). Nevertheless, until today no isoform-specific inhibitors could be validated.

The $\alpha_2\delta$ subunit: Four genes (*Cacna2d1-4*) encode for $\alpha_2\delta$ subunits ($\alpha_2\delta$ -1 to $\alpha_2\delta$ -4). They display distinct tissue distribution and three isoforms ($\alpha_2\delta$ -1 to -3) are strongly expressed in the developing and mature CNS (Arikkath and Campbell 2003; Schlick et al. 2010). Mature $\alpha_2\delta$ subunits consist of posttranslationally cleaved highly glycosylated α_2 and δ peptides (Fig. 1B), which are associated to each other by a disulfide bond (Calderon-Rivera et al. 2012). Glycosylation of $\alpha_2\delta$ subunits is required for the functional membrane expression of calcium channels, as deglycosylation and glycosylation-site-directed mutagenesis resulted in strongly reduced current densities without affecting the kinetic properties (Gurnett et al. 1996; Sandoval et al. 2004). $\alpha_2\delta$ subunits are most likely linked to the plasma membrane *via* GPI-anchors (Davies et al. 2010). Alternatively, the δ subunit may constitute a single-pass membrane protein (Robinson et al. 2011). The vast majority of the $\alpha_2\delta$ protein is extracellular, ideally situated to interact with constituents of the extracellular matrix or extracellularly exposed proteins. In the domain structure of α_2 a von Willebrand factor type A (VWA) domain and two Cache domains were identified by sequence homology in all $\alpha_2\delta$ subunits (Fig. 1B; Anantharaman and Aravind 2000; Canti et al. 2005; Davies et al. 2007). VWA-domains are found in a variety of extracellular matrix proteins and integrin receptors and are well known for their role in cell-cell adhesion involving a metal ion-dependent adhesion site (MIDAS). The integrity of the MIDAS motif in $\alpha_2\delta$ -2 has been shown to be necessary for calcium current enhancement and Ca_V channel trafficking (Canti et al. 2005). It has been hypothesized that these domains may be regulated by small endogenous ligands, such as the amino acid isoleucine (reviewed in Dooley et al. 2007), and that they are involved in binding the anti-epileptic and anti-allodynic drugs gabapentin (GBP) and pregabalin (PG) (Davies et al. 2007), which have also proven clinical efficacy in the treatment of generalized anxiety disorders.

2.2. Basic principles of neuronal functions mediated by voltage-gated calcium channels

The strong buffering of the second messenger calcium requires a close temporal and spatial vicinity of effector proteins to the calcium channel complex. Accordingly, neuronal Ca_V s are closely associated with upstream modulators, like protein kinases and phosphatases, and downstream effectors, as well as adapter and scaffold proteins. In neurons two highly specialized complexes have been intensively characterized: the synaptic vesicle fusion apparatus mediating excitation-secretion coupling and the postsynaptic calcium channel complex mediating excitation-transcription coupling (Fig. 2). At the synaptic vesicle fusion apparatus presynaptic calcium channels (mainly $\text{Ca}_V2.1$ and $\text{Ca}_V2.2$) are located in the close vicinity of the calcium sensor synaptotagmin, which triggers vesicle fusion and thereby neurotransmitter release. In the postsynaptic compartment activation of L-type channels ($\text{Ca}_V1.2$ and $\text{Ca}_V1.3$) initiates a signaling cascade to the nucleus that regulates gene expression. In both specialized compartments calcium channels need to be tightly organized in macromolecular complexes with upstream and downstream signaling molecules (Fig. 2B and C). The molecular organization of these signaling complexes is expected to be

influenced by the subunit composition of the Ca_V complex in several ways. First, modulation of current properties (activation and inactivation kinetics, voltage-dependence) by auxiliary subunits will affect the local calcium transient and thereby downstream signaling. Second, a role of auxiliary Ca_V subunits in channel trafficking, targeting, and scaffolding will affect the composition of the entire complex and thereby determine specificity. Because different subunit isoforms and splice variants may differ with respect to their modulatory properties, protein-protein interactions, and subcellular targeting, the diversity of the auxiliary subunits may determine specific cellular functions in neurons. Therefore, in order to understand the role of the various distinct auxiliary Ca_V subunits in neurons it is necessary to address 1) which subunit isoforms and splice variants are actual components of specific signaling complexes, 2) whether different isoforms and splice variants serve distinct functions, 3) whether and to what degree can specific functions be compensated by other isoforms, and finally 4) do auxiliary subunits also act in a molecular context which is independent of the calcium channel complex.

2.3. Establishing and maintaining signaling specificity

In order to elucidate the physiological neuronal functions of calcium channels and their auxiliary subunits, understanding the specificity of Ca_V subunit interactions and complex formation in native differentiated cell systems is a prerequisite. Three distinct mechanisms may contribute to the specificity of neuronal Ca_V complexes (Obermair and Flucher 2013). First, the calcium subunit complement in a specific cell type at a given time will yield the formation of particular complexes. Such preferential expression patterns of α_1 , β , and $\alpha_2\delta$ isoforms can indeed be observed in skeletal and cardiac muscle as well as highly specialized neuronal cell types like retina photoreceptor cells (Knoflach et al. 2013). In the CNS various brain regions including cortex, hippocampus, cerebellum, and even single cell types like cultured hippocampal pyramidal cells simultaneously express physiologically relevant mRNA levels of five out of seven high-voltage activated α_1 subunits, all four β subunit isoforms, and three of four $\alpha_2\delta$ subunits (Schlick et al. 2010). The cerebellum, however, represents one notable exception as it preferentially expresses one set of calcium channel subunits ($\text{Ca}_V2.1/\beta_4/\alpha_2\delta-2$) (Ludwig et al. 1997; Brodbeck et al. 2002; Schlick et al. 2010). Altogether, in the majority of brain regions a restricted expression of auxiliary subunit isoforms may not be the prime strategy for establishing specific Ca_V complexes.

Second, distinct subcellular targeting properties of individual subunits or splice variants and/or possible interactions with other proteins may also yield specificity. We have recently identified such a mechanism for splice variants of the auxiliary β_4 subunits. Whereas all β_4 splice variants could increase the expression of presynaptic $\text{Ca}_V2.1$ by directly interacting with the channel, only β_4 splice variants targeted into the nucleus (β_{4b} , β_{4a}) did specifically repress expression of synaptic proteins including $\text{Ca}_V2.1$ in a channel-independent manner (Etemad et al. 2014a, 2014b). Recently the differential up-regulation of an $\alpha_2\delta-1$ splice variant in DRG neurons after spinal nerve ligation was identified (Lana et al. 2014). This finding supports a functional heterogeneity of splice variants also with $\alpha_2\delta$ subunits.

Finally it is also possible that specific stable complexes may not exist in all neuronal compartments and calcium channels could be regulated by reversible interactions with pools

of functionally diverse β or $\alpha_2\delta$ subunits. An elegant FRAP study in skeletal muscle cells recently confirmed the presence of a stable calcium channel complex between the homologous $\text{Ca}_V1.1$ and β_{1a} isoforms, while the heterologous β_{2a} and β_{4b} subunits formed dynamic complexes with the channel (Campiglio et al. 2013). The existence of a mobile pool of the L-type channel $\text{Ca}_V1.2$ in hippocampal neurons may be a first hint towards regulation of neuronal Ca_V s by dynamic subunit interactions (Di Biase et al. 2011).

3. Neuronal roles of auxiliary $\alpha_2\delta$ subunits

3.1. Channel trafficking and current modulation

The classical roles of auxiliary $\alpha_2\delta$ subunits on voltage-gated calcium channels, namely regulating the functional membrane expression and modulating the calcium currents, are widely recognized (Fig. 3A and B). When heterologously expressed all $\alpha_2\delta$ isoforms can enhance the functional membrane expression of α_1 subunits (reviewed in Arikath and Campbell 2003; Davies et al. 2007; Obermair et al. 2008). $\alpha_2\delta$ -1 co-expression studies in tsA201 cells additionally identified a role in modulating the biophysical channel properties such as activation and inactivation kinetics as well as the voltage-dependence of activation (Felix et al. 1997). Similar to $\alpha_2\delta$ -1 also $\alpha_2\delta$ -3 increased the functional membrane expression and altered the biophysical properties of L-type calcium channels (Klugbauer et al. 1999). In contrast, expression of $\alpha_2\delta$ -2 in *Xenopus* oocytes did not reveal biophysical modulation of $\text{Ca}_V1.2$ and $\text{Ca}_V2.2$ channels, although it was also found to increase the channels' current density (Gao et al. 2000; Brodbeck et al. 2002). $\alpha_2\delta$ -4 is the least well studied isoform among $\alpha_2\delta$ subunits with respect to its modulatory effects on Ca_V s. In HEK293 it has been shown to increase calcium influx *via* $\text{Ca}_V1.2$ channels (Qin et al. 2002); however, a detailed functional characterization is still missing. Altogether existing evidence indicates some isoform specificity in modulating the biophysical current parameters, while increasing the functional membrane expression of Ca_V s seems to be a rather general mechanism. On the other hand isoform specificity in channel trafficking has been described in non-neuronal excitable cells. In skeletal muscle cells were only one isoform ($\alpha_2\delta$ -1) is expressed, experiments utilizing an $\alpha_2\delta$ -1 shRNA approach demonstrated that this subunit is not essential for targeting of calcium channels or for their primary physiological role in activating skeletal muscle excitation-contraction coupling (Obermair et al. 2005). However, $\alpha_2\delta$ -1 was identified as the major determinant of the characteristic L-type calcium current kinetics (Obermair et al. 2005; Tuluc et al. 2007). Together this suggests that $\alpha_2\delta$ -1 does not regulate the membrane trafficking and targeting of L-type calcium channels in their native environment. In consistence with this hypothesis $\alpha_2\delta$ -1 had only a small trafficking effect on $\text{Ca}_V1.2$ in a model system for cardiac myocytes (Tuluc et al. 2007), whereas it strongly enhanced functional expression of neuronal N- ($\text{Ca}_V2.2$) and P/Q-type ($\text{Ca}_V2.1$) channels (Obermair et al. 2008). In neurons of the CNS, where three $\alpha_2\delta$ isoforms ($\alpha_2\delta$ -1, -2, -3) are expressed, co-expression of all three subunits with P/Q or N-type channels causes an increase in current density (Davies et al. 2007). Thus regulating membrane trafficking and thereby calcium channel abundance may be the primary mechanism for modulating specific neuronal functions, for example synaptic transmission, by $\alpha_2\delta$ subunits. This is supported by reduced presynaptic $\text{Ca}_V2.1$ targeting as well as reduced synaptic release probability in neurons where $\alpha_2\delta$ -1 was knocked down by shRNA (Hoppa et al. 2012). On the other hand,

the largely mild CNS phenotypes of the individual knockout mice (see below) suggest compensatory actions of $\alpha_2\delta$ isoforms. The propensity of individual $\alpha_2\delta$ -isoforms to regulate channel targeting is one important aspect in controlling Ca_V s, the other aspect is their capacity to fine tune the current kinetics of individual calcium channels. This was not only observed for skeletal and cardiac muscle Ca_V s (see above). Constitutive overexpression of $\alpha_2\delta$ -1 in neuronal tissues, for instance, resulted in enhanced currents as well as altered kinetics and voltage-dependence of activation in sensory neurons (Li et al. 2006).

3.2. $\alpha_2\delta$ subunits as targets for gabapentinoid drugs

Although the chemical backbone of GBP and PG was derived from GABA the mechanism of action of both drugs was initially unclear. The only outcome was that they did not have any notable effect on GABA receptors, metabolism, or transport (reviewed in Taylor et al. 2007; Silverman 2008; Bauer et al. 2010). However, both drugs were shown to reduce the cellular influx of calcium *via* Ca_V s in synaptosome fractions and hippocampal synapses (Fink et al. 2002; van Hooft et al. 2002). Accordingly it was a surprising result as it turned out that the 3H-gabapentin binding site corresponded to $\alpha_2\delta$ -1 and finally a selective binding to $\alpha_2\delta$ -1 and $\alpha_2\delta$ -2 was confirmed by the availability of newly established mouse models (see below). Both GBP and PG were found to be effective treatments for various forms of neuropathic pain and are also used as an adjunctive therapy of certain forms of epilepsy. The effect of these drugs in neuropathic pain was found to be state-dependent in that they have little effect on acute pain perception in animals or humans, but they are effective in chronic neuropathic pain. In line with this long term effects GBP inhibits calcium currents when applied chronically, both in heterologous expression systems and in dorsal root ganglion neurons (e.g. Hendrich et al. 2008). The most likely explanation for this effect is that chronic GBP treatment interferes with Ca_V trafficking to the cell surface as well as to presynaptic terminals (Bauer et al. 2009; Tran-Van-Minh and Dolphin 2010). Besides affecting trafficking during chronic application, these drugs have also been shown to exert acute effects on the current amplitude and the biophysical properties of calcium currents as well as on Ca_V -dependent functions, such as synaptic transmission (e.g. Uchitel et al. 2010; Farrell et al. 2014). However, acute effects of GBP and PG have not been observed in all studies and may depend on the composition of the calcium channel complex (Martin et al. 2002).

3.3. $\alpha_2\delta$ subunits and synapse formation

Over the recent years accumulating evidence suggests an important role of $\alpha_2\delta$ subunits in synapse formation (Fig. 3C). $\alpha_2\delta$ -1 has been shown to act as a receptor for thrombospondin, an astrocyte-secreted protein that promotes CNS synaptogenesis (Eroglu et al. 2009). In this study overexpression of $\alpha_2\delta$ -1 strongly promoted, and shRNA knockdown inhibited excitatory synapse formation in cultured retinal ganglion cells. *Drosophila* mutants of the $\alpha_2\delta$ -3 homologue (straightjacket; stj) show defects in presynaptic calcium channel localization and synaptic function (Dickman et al. 2008), which also coincides with a failure in normal synapse development in motoneurons (Kurshan et al. 2009). Interestingly, this study provided evidence that the defect in synapse formation was independent from the *Drosophila* pore forming α_1 subunit (cacophony), because in contrast to the stj mutants, cacophony null mutants did not show a defect in synapse formation. $\alpha_2\delta$ -2 mutant (ducky, ducky^{2J}) and knockout mice display altered Purkinje cell morphology and reduced calcium

currents (Barclay et al. 2001; Ivanov et al. 2004; Donato et al. 2006), which suggests a role in maintaining normal cellular physiology and likely also the morphology of synapses. A spontaneous mouse mutation of $\alpha_2\delta$ -4 (Cacna2d4) causes structural and functional abnormalities of retinal ribbon synapses associated with the loss of rods (Wycisk et al. 2006a). Unc-2 and unc-36, the *C. elegans* homologues of Ca_v2 and $\alpha_2\delta$, respectively, were identified as regulators of *C. elegans* synaptogenesis (Caylor et al. 2013). While for unc-2 the study revealed a role in regulating the dynamic changes in the size and morphology of synapses that occur during development, unc-36 was demonstrated to regulate presynaptic morphology. $\alpha_2\delta$ -1 upregulation in a nerve injury model for chronic pain induced abnormal excitatory synapse formation and enhanced neurotransmitter release (Li et al. 2014). Finally, $\alpha_2\delta$ -3 knockout mice have a defect in presynaptic $\text{Ca}_v2.1$ channel targeting and synapse formation of auditory nerve fiber terminals contacting cochlear nucleus bushy cells (Pirone et al. 2014). Taken together, evidence is accumulating suggesting a role of $\alpha_2\delta$ subunits in synapse formation, which may be in part or entirely independent of the calcium channel complex. The mechanistic background for an independent function of $\alpha_2\delta$ subunits could be that these proteins protrude far into the extracellular space and therefore have the potential to contribute to signaling events that coordinate synaptic development.

3.4. Insights from animal models and human disease mutations

Within the past years, there has been great progress in the generation and description of mouse models with targeted deletions and spontaneous mutations for all $\alpha_2\delta$ subunits. These knockout and mutant mice display various disease phenotypes, a part of which share similarities with recently identified human diseases linked to $\alpha_2\delta$ subunits. The involvement of $\alpha_2\delta$ subunits in disease gives important insight into possible functions of the distinct isoforms (summarized in Tab. 1).

$\alpha_2\delta$ -1: As $\alpha_2\delta$ -1 (and $\alpha_2\delta$ -2) were identified targets for GBP and PG, the need for mouse models was soon recognized for studying the involvement of the distinct $\alpha_2\delta$ s in neuropathic pain and epilepsy. By generating a knock-in mouse expressing a mutated $\alpha_2\delta$ -1, that is not able to bind GBP and PG, Field et al. (2006) revealed $\alpha_2\delta$ -1 being the *in vivo* target for gabapentinoid drugs. This was ultimately confirmed by the lack of high affinity GBP binding in the brain of a conventional $\alpha_2\delta$ -1 knockout mouse (Fuller-Bicer et al. 2009). As previously expected from studies in skeletal and cardiac myocytes (Obermair et al. 2005; Tuluc et al. 2007), these mice display decreased cardiac L-type currents with altered activation kinetics. In somatosensory neurons $\alpha_2\delta$ -1 null mice showed a reduced $\text{Ca}_v2.2$ level and calcium channel current density, which was associated with a deficit in mechanical and cold sensitivity (Patel et al. 2013). Nevertheless, despite the widespread and predominant expression of $\alpha_2\delta$ -1 in the brain, so far neither gross-anatomical alterations nor signs for neuronal disease have been identified. Very recently disruption and deletion of the CACNA2D1 gene in three human patients with epilepsy and intellectual disability has been described (Vergult et al. 2014). Interestingly similar gene aberrations have also been identified in healthy individuals; therefore the causal relation of $\alpha_2\delta$ -1 disruption and human disease is still elusive. Senatore et al. (2012) identified an interaction of $\alpha_2\delta$ -1 with mutant prion proteins, putting forward the idea that disrupted neurotransmission in prion diseases may be linked to impaired synaptic Ca_v trafficking via $\alpha_2\delta$ subunits. Taken together the role

of $\alpha_2\delta$ -1 for CNS disease remains unclear and the resulting phenotypes and severities may be associated with alterations in the overall neuronal excitability, which can be influenced by a variety of other factors.

$\alpha_2\delta$ -2: There are several strains of naturally occurring mice with mutations in the *Cacna2d2* gene (ducky, ducky^{2j}, and entla) (Barclay et al. 2001; Brodbeck et al. 2002; Brill et al. 2004; Donato et al. 2006) as well as a targeted $\alpha_2\delta$ -2 knockout mouse (Ivanov et al. 2004), which result in a loss of the full-length $\alpha_2\delta$ -2 protein (ducky, ducky^{2j}, knockout) or a structurally altered protein (entla). Affected homozygous mice show a decreased life span, infertility, and reduced body size when compared to heterozygous or wildtype littermates. The mice suffer from ataxia, paroxysmal dyskinesia, as well as epilepsy. $\alpha_2\delta$ -2 null mice (ducky, ducky^{2j}, knockout) also exhibit altered cerebellar purkinje cell morphology and reduced calcium currents, as well as brainstem dysgenesis and spinal cord and myelination defects. In humans *CACNA2D2* has been discussed as a potential tumor suppressor gene (Hesson et al. 2007) and a single nucleotide polymorphism (SNP)-based study identified *CACNA2D2* as a candidate gene in childhood absence epilepsy (Chioza et al. 2009). Indeed a point mutation in *CACNA2D2* resulting in reduced current density and slowed inactivation in neuronal calcium channels has recently been associated with early infantile epileptic encephalopathy (Edvardson et al. 2013). Pippucci et al. (2013) identified a frameshift mutation in *CACNA2D2* in a patient with epilepsy, dyskinesia, cerebellar atrophy, psychomotor delay and dysmorphic features, a disease strikingly similar to the above-mentioned phenotypes of $\alpha_2\delta$ -2 mouse models.

$\alpha_2\delta$ -3: Mice with a targeted deletion of the *Cacna2d3* gene and the concomitant insertion of a bacterial β -galactosidase under its promoter were generated by Deltagen, Inc. These mice exhibit deficits in nociceptive pain processing and a delay in inflammatory heat hyperalgesia (Neely et al. 2010). Interestingly, $\alpha_2\delta$ -3 knockout mice display a reduced acoustic startle reflex and distorted auditory brainstem responses, which are likely caused by defects in presynaptic $Ca_v2.1$ channel targeting and synapse formation of auditory nerve fiber terminals contacting cochlear nucleus bushy cells (Pirone et al. 2014). *Drosophila* embryos lacking the $\alpha_2\delta$ -3 homologue straightjacket display altered heat nociception, a decrease in synaptic transmission and Ca_v2 channel abundance, as well as morphologically altered synaptic structure (Dickman et al. 2008; Kurshan et al. 2009; Neely et al. 2010). The *C. elegans* $\alpha_2\delta$ -3 mutant *unc-36* revealed a role in Ca_v2 (*unc-2*) channel trafficking (Saheki and Bargmann 2009) which is associated with altered presynaptic morphology (Caylor et al. 2013). However, so far the only link between $\alpha_2\delta$ -3 and human disease is the identification of SNPs within the large genomic region of *CACNA2D3* in patients with reduced sensitivity to acute noxious heat and chronic back pain (Neely et al. 2010).

$\alpha_2\delta$ -4: Although expression of $\alpha_2\delta$ -4 is negligible in CNS neurons (Schlick et al. 2010), $\alpha_2\delta$ -4 is the major $\alpha_2\delta$ subunit in retinal photoreceptor cells (Knoflach et al. 2013). Mice with a spontaneous mutation of *Cacna2d4* resulting in a truncated $\alpha_2\delta$ -4 protein show structural and functional abnormalities in mouse ribbon synapses associated with the loss of rods (Wycisk et al. 2006a). A similar phenotype was reported in humans, where a comparable mutation of the *CACNA2D4* gene causes slowly progressing cone dystrophy

associated with night blindness (Wycisk et al. 2006b). In a genome-wide association study a rare partial deletion in CACNA2D4 in two patients with late onset bipolar disorder was identified and also other genetic studies provide links between the $\alpha_2\delta$ -4 gene and psychiatric disorders (Van Den Bossche et al. 2012; see discussion therein). Any causal and mechanistic explanations for these associations remain as for now speculative, but may involve the expression of $\alpha_2\delta$ -4 in a specific and low abundant CNS cell type. Other causes could be provided by the role of $\alpha_2\delta$ -4 in vision or the close genetic proximity to the CACNA1C gene encoding for $\text{Ca}_V1.2$, which was shown to be associated with psychiatric disorders (Splawski et al. 2004).

4. Conclusion and outlook

Over the last years progress in unraveling the specific neuronal functions of neuronal $\alpha_2\delta$ subunits has been made at several levels. The availability of novel knock-out and mutant animals together with sophisticated heterologous expression studies in a variety of cell systems opened the possibility studying the specific roles of $\alpha_2\delta$ subunit isoforms. The findings showed that all $\alpha_2\delta$ subunits have classical roles dependent on the Ca_V complex, such as the modulation of membrane trafficking or current properties. Interestingly, more recent studies suggested functions of $\alpha_2\delta$ subunits in synapse formation, which may be in part or entirely independent of the Ca_V complex. Because, however, any defect or loss-of-function of $\alpha_2\delta$ subunits will inevitably affect the entire calcium channel complex, a clear distinction between independent or dependent functions is inherently difficult.

An emerging common feature in loss-of-function models of single $\alpha_2\delta$ isoforms is that the loss of one isoform only induces a strong phenotype in cells or tissues expressing predominantly this respective isoform. Such a pattern can be observed in the retina ($\alpha_2\delta$ -4; Wycisk et al. 2006a), in retinal ganglion cells ($\alpha_2\delta$ -1; Eroglu et al. 2009), in auditory nerves ($\alpha_2\delta$ -3; Pirone et al. 2014), and is also supported by studies in invertebrates (Kurshan et al. 2009; Caylor et al. 2013). Quite contrary, in CNS brain regions or neurons which simultaneously express three different $\alpha_2\delta$ isoforms the consequences of loss-of-function of a particular isoform may be compensated by the other isoforms thereby obscuring any potential phenotype. Thus, the severe consequences of $\alpha_2\delta$ -2 mutations in animal models and human disease are likely caused by the predominant expression of this particular isoform in the cerebellum. There the low protein levels of $\alpha_2\delta$ -1 and $\alpha_2\delta$ -3 do not suffice to compensate the loss of $\alpha_2\delta$ -2. Alternatively, $\text{Ca}_V2.1$ may exclusively form a complex with the auxiliary $\alpha_2\delta$ -2 and β_4 subunits. Another possibility is that $\alpha_2\delta$ -2 itself mediates unique channel-independent functions, which cannot be compensated by the other isoforms. This possibility can be tested by analyzing calcium channels and Ca_V -dependent neuronal functions in cortical neurons from $\alpha_2\delta$ -2 knockout mice, as these neurons predominantly express $\alpha_2\delta$ -1 and $\alpha_2\delta$ -3 (Schlick et al. 2010). This endeavor is, however, complicated by the severe overall phenotype of $\alpha_2\delta$ -2 knockout and mutant mice. In a nutshell the available animal models, experimental co-expression studies, as well as identified human disease mutations at the moment do not provide a clear cut answer as to whether the individual $\alpha_2\delta$ isoforms have specific roles in CNS neurons.

Taken together, for the future the main challenge in elucidating the roles of neuronal $\alpha_2\delta$ subunits will be sorting out the functions of individual isoforms in cells and tissues simultaneously expressing more than one isoform. In order to clearly distinguish between potential redundant and specific functions it will be necessary to generate knockout mouse models lacking different combinations of $\alpha_2\delta$ isoforms (e.g. double knockout models). Such mouse models need to be complemented by native neuronal expression systems lacking two if not all three $\alpha_2\delta$ isoforms for studying the consequences on pre- and postsynaptic Ca_V -dependent functions. Characterization of the behavioral phenotype and the brain anatomy of such mouse models as well as analyzing neuronal functions could strongly contribute to a better understanding of the molecular mechanism of $\alpha_2\delta$ signaling and potentially uncover novel pathophysiological mechanisms.

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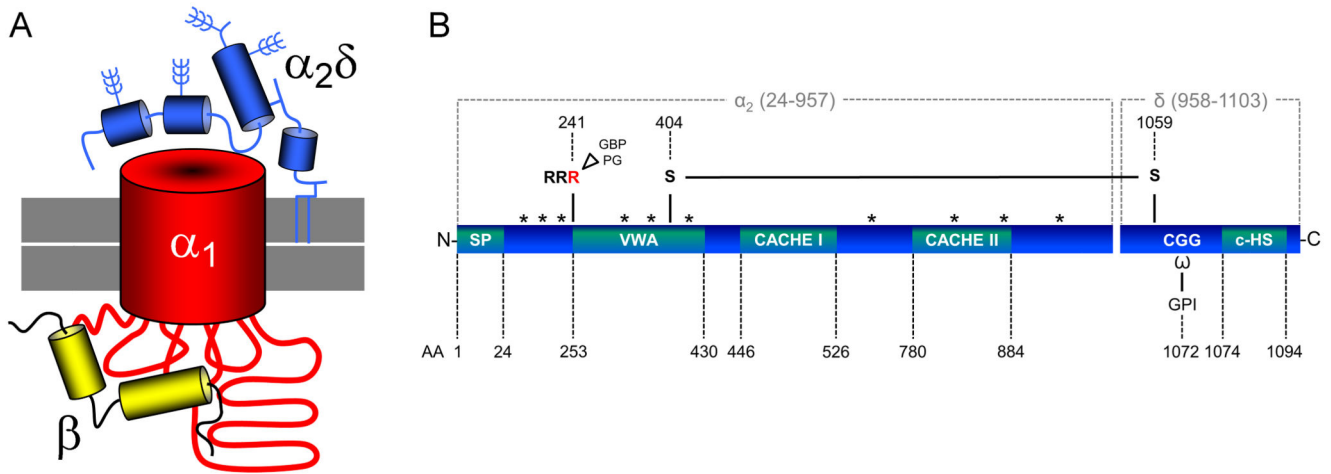


Figure 1.

A. The voltage-gated calcium channel complex. Voltage-gated calcium channels are composed of a transmembrane pore-forming α_1 subunit (red) which forms a macromolecular complex together with extracellular $\alpha_2\delta$ (blue) and cytoplasmic β (yellow) subunits. The β subunit binds the intracellular I-II linker of α_1 with high affinity. The $\alpha_2\delta$ subunit consists of posttranslationally cleaved highly glycosylated α_2 and δ peptides, which are associated to each other by a disulfide bond and are most likely linked to the plasma membrane (indicated in grey) *via* a GPI-anchor. Note that the folding structure of the individual subunits is simplified. **B.** Domain structure of auxiliary $\alpha_2\delta$ subunits. Amino acid positions refer to the mouse $\alpha_2\delta$ -1 isoform [uniprot: O08532- CA2D1_MOUSE], however all isoforms have a similar topology. Amino acid residues 1–24 encode the signal peptide (SP). The α_2 peptide contains a von Willebrand factor type A (VWA) domain and two sequence stretches homologous to extracellular domains of bacterial chemosensing proteins (Cache I and II). In $\alpha_2\delta$ -1 and $\alpha_2\delta$ -2 an arginine (RRR) motif proximal to the VWA domain represents the potential gabapentin (GBP) and pregabalin (PG) binding site. Two cysteine residues (AA 404 and 1059) were identified to be important for the formation of an intermolecular disulfide bond between α_2 and δ . The δ peptide contains predicted ω amino acids, to which the GPI anchor can attach, and a C-terminal hydrophobic sequence (c-HS). Potential glycosylation sites are indicated by asterisks (*).

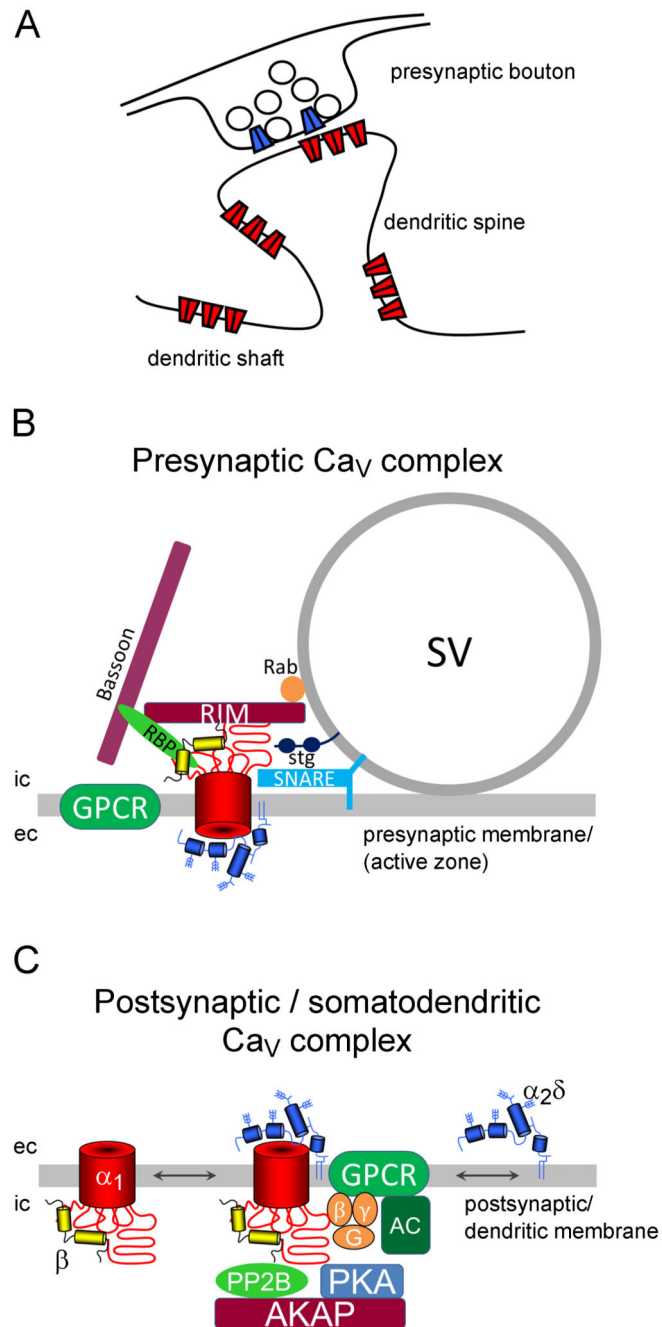
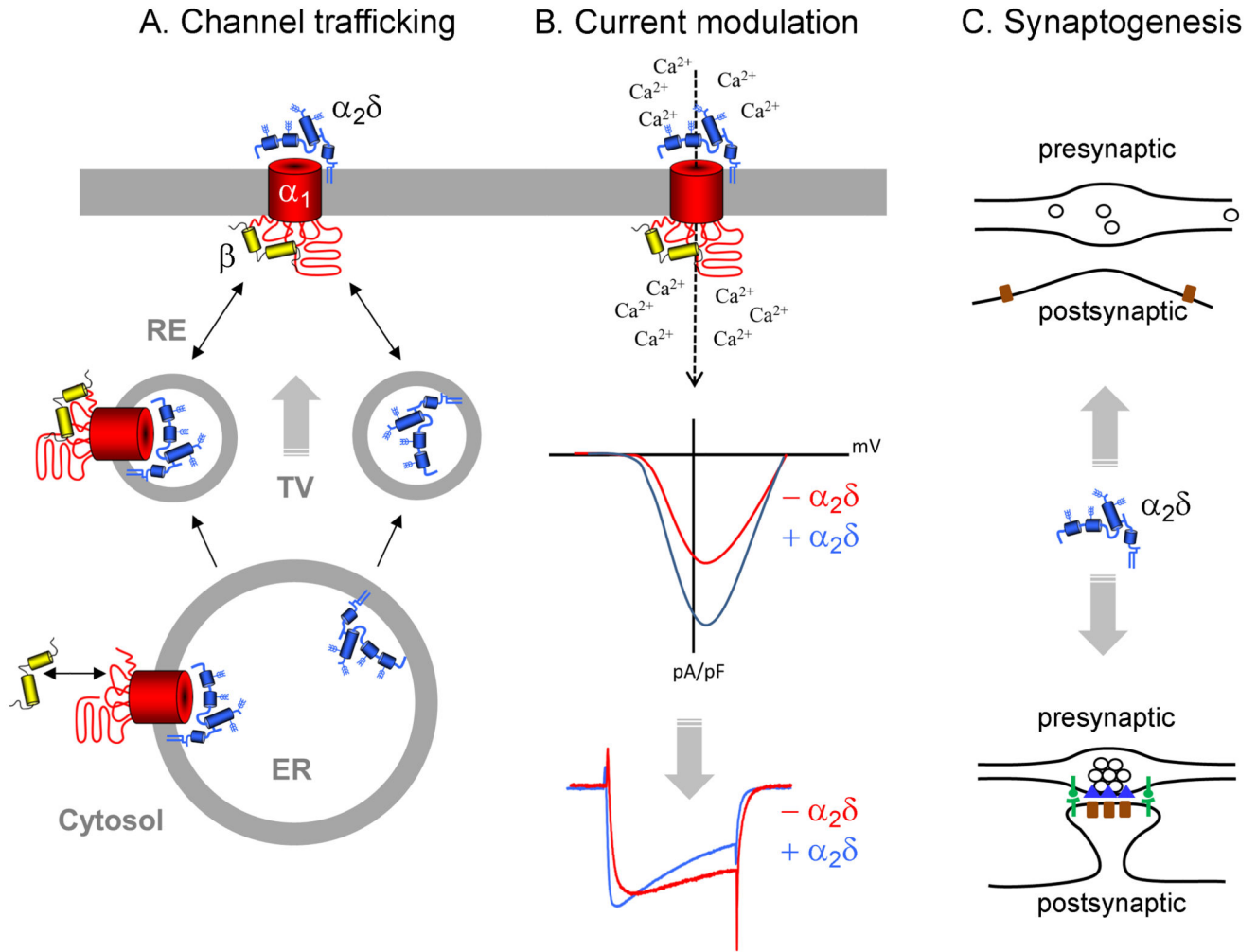


Figure 2. Neuronal calcium channel complexes. **A.** In neurons voltage-gated calcium channels are located in presynaptic boutons, where they trigger neurotransmitter release, and in postsynaptic as well as extrasynaptic positions along dendrites and dendritic spines (Obermair et al. 2004; Jenkins et al. 2010). **B.** In the presynaptic active zone calcium channels are organized in a dense network of presynaptic proteins (depicted are RIM, RBP, Rab, bassoon, SNARE complex) and G-protein coupled receptors (GPCR) in the vicinity of the calcium sensor synaptotagmin (stg). Together this macromolecular complex orchestrates

the tight regulation of synaptic vesicle (SV) fusion and thus neurotransmitter release (reviewed in Südhof 2012). C. In the postsynaptic/somato-dendritic compartment L-type channels can be found in complexes with G-protein coupled receptors (GPCR, e.g. the β_2 adrenergic receptor), an adenylyl cyclase (AC), protein kinases (PKA) and phosphatases (PP2B), and scaffolding proteins (e.g. AKAP150) (reviewed in Dai et al. 2009). Downstream signaling initiates a signaling cascade which leads to the modulation of transcription such as CREB or NFATc4. Lateral mobility of L-type calcium channels in the plasma membrane also suggests regulation via the association or dissociation with distinct signaling complexes (Di Biase et al. 2011). Considering the membrane diffusion of GPI-anchored proteins, such a mechanism may also apply to $\alpha_2\delta$ subunits. ic, intracellular; ec, extracellular.

**Figure 3.**

Sketch summarizing the neuronal functions of auxiliary $\alpha_2\delta$ subunits. **A.** Channel trafficking: The export from the endoplasmic reticulum (ER) is mediated by β subunits; however, $\alpha_2\delta$ subunits can further enhance the trafficking. This may involve trafficking from the endoplasmic reticulum (ER) via the Golgi apparatus in transport vesicles (TV), recycling from recycling endosomes (RE), or stabilization of the channel complex at the plasma membrane. $\alpha_2\delta$ subunits can also be transported to the plasma membrane independent of the α_1 subunit. **B.** Current modulation: Besides their role in channel trafficking $\alpha_2\delta$ subunits are important modulators of the calcium current. $\alpha_2\delta$ subunits can shift the voltage-dependence of channel activation to more negative potentials (IV curve, middle graph) and can modify the activation and inactivation kinetics (current traces, lower graph). This may be of particular relevance for somato-dendritic L-type calcium channels. **C.** Synaptogenesis: Accumulating evidence suggests an important role of neuronal $\alpha_2\delta$ subunits in synapse formation. Whether $\alpha_2\delta$ s are necessary for the initial contact between the axon and the corresponding postsynaptic membrane (top) or for the differentiation into a fully aligned and mature synapse (bottom) remains to be answered.

Table 1

 $\alpha_2\delta$ subunit isoforms and mouse and human disease phenotypes

Mouse		Human		
Isoform	Mouse models	Phenotype	Potential cause	Disease
$\alpha_2\delta$ -1	knock-in ¹ (GBP insensitive)	no known CNS phenotype ¹	aberrations of CACNA2D1 ⁵	epilepsy and intellectual disability ⁵
	knockout ²	reduced pain sensitivity ³ , no known CNS phenotype ¹		
	$\alpha_2\delta$ -1 over-expressing ⁴	hyperalgesia, tactile allodynia ⁴		
$\alpha_2\delta$ -2	spontaneous loss-of-function mutations ^{6,7,8,9} (<i>ducky</i> , <i>ducky</i> ^{2j} , <i>entla</i>), knockout ¹⁰	ataxia, epilepsy and paroxysmal dyskinesia ^{6,7,8,9,10}	homozygous mutations (point ¹¹ and frameshift ¹²)	epileptic encephalopathy ^{11,12}
			genomic deletion of CACNA2D2 ^{13,14}	potential tumor suppressor gene ^{13,14}
			single-nucleotide polymorphisms (SNPs) ¹⁵	candidate gene in childhood absence epilepsy ¹⁵
$\alpha_2\delta$ -3	knockout ¹⁶	deficits in pain ¹⁶ and auditory/acoustic startle processing ¹⁷	SNPs ¹⁶	reduced sensitivity to acute noxious heat and chronic back pain ¹⁶
			splice site mutation in CACNA2D3 ¹⁸	autism spectrum disorders ¹⁸
$\alpha_2\delta$ -4	spontaneous mutation (premature stop) ¹⁹	structural and functional abnormalities in ribbon synapses, loss of rods ¹⁹	mutation (premature stop) of CACNA2D4 ²⁰	slowly progressive cone dystrophy and night blindness ²⁰
			partial deletion of CACNA2D4 ²¹	psychiatric disorders ²¹

¹Field et al. 2006,²Fuller-Bicer et al. 2009,³Patel et al. 2013,⁴Li et al. 2006,⁵Vergult et al. 2014,⁶Barclay et al. 2001,⁷Brodbeck et al. 2002,⁸Brill et al. 2004,⁹Donato et al. 2006,¹⁰Ivanov et al. 2004,¹¹Edvardson et al. 2013,¹²Pippucci et al. 2013,¹³Carboni et al. 2003,

¹⁴Hesson et al. 2007,

¹⁵Chioza et al. 2009,

¹⁶Neely et al. 2010,

¹⁷Pirone et al. 2014,

¹⁸Iossifov et al. 2012,

¹⁹Wycisk et al. 2006a,

²⁰Wycisk et al. 2006b,

²¹Van Den Bossche et al. 2012