



Neuronal $\alpha_2\delta$ proteins and brain disorders

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Abstract

$\alpha_2\delta$ proteins are membrane-anchored extracellular glycoproteins which are abundantly expressed in the brain and the peripheral nervous system. They serve as regulatory subunits of voltage-gated calcium channels and, particularly in nerve cells, regulate presynaptic and postsynaptic functions independently from their role as channel subunits. $\alpha_2\delta$ proteins are the targets of the widely prescribed anti-epileptic and anti-allodynic drugs gabapentin and pregabalin, particularly for the treatment of neuropathic pain conditions. Recently, the human genes (CACNA2D1–4) encoding for the four known $\alpha_2\delta$ proteins (isoforms $\alpha_2\delta$ -1 to $\alpha_2\delta$ -4) have been linked to a large variety of neurological and neuropsychiatric disorders including epilepsy, autism spectrum disorders, bipolar disorders, schizophrenia, and depressive disorders. Here, we provide an overview of the hitherto identified disease associations of all known $\alpha_2\delta$ genes, hypothesize on the pathophysiological mechanisms considering their known physiological roles, and discuss the most imminent future research questions. Elucidating their specific physiological and pathophysiological mechanisms may open the way for developing entirely novel therapeutic paradigms for treating brain disorders.

Keywords Neurological disease · Autism · Schizophrenia · Epilepsy · Synapses · CACNA2D1–4

Introduction

$\alpha_2\delta$ proteins are membrane-anchored extracellular glycoproteins which have initially been identified as subunits of voltage-gated calcium channels (VGCCs). An increasing number of studies, however, suggest functions independent of the calcium channel complex. $\alpha_2\delta$ proteins are the targets of the widely prescribed anti-epileptic and anti-allodynic drugs gabapentin and pregabalin and have been linked to a large variety of diseases. In this review, we provide an overview of the known and putative associations of $\alpha_2\delta$ genes and

proteins with human neurological disorders. Furthermore, we discuss channel-dependent and channel-independent mechanisms of potential relevance for the respective pathophysiological mechanisms.

$\alpha_2\delta$ genes and proteins

In the human genome, four genes (CACNA2D1–4) code for four $\alpha_2\delta$ proteins ($\alpha_2\delta$ -1 to $\alpha_2\delta$ -4), which give rise to multiple transcripts as the result of alternative splicing [31, 60]. The genes contain 38 or 39 exons, and the distribution of exons over the gene is similar between $\alpha_2\delta$ -1 and $\alpha_2\delta$ -2 (Fig. 1) but considerably different in $\alpha_2\delta$ -3 and $\alpha_2\delta$ -4 (Fig. 2). Mature $\alpha_2\delta$ proteins lack the co-translationally cleaved N-terminal signal peptide and are highly glycosylated extracellular proteins of 140 to 170 kDa. They are posttranslationally cleaved into α_2 and δ moieties, which are covalently bound through disulfide bonds [13, 27, 126]. $\alpha_2\delta$ proteins are most likely attached to the plasma membrane via glycosylphosphatidylinositol (GPI) anchors [26]; alternatively, however, the δ polypeptide chain may form a transmembrane α -helix [91]. Even though all $\alpha_2\delta$ subunits contain well-known protein domains including a von Willebrand factor A (VWA) domain, the detailed structure is still under debate. As of today, the most precise estimation of $\alpha_2\delta$ protein structure is based on a cryogenic electron microscopy (cryo-EM) study of

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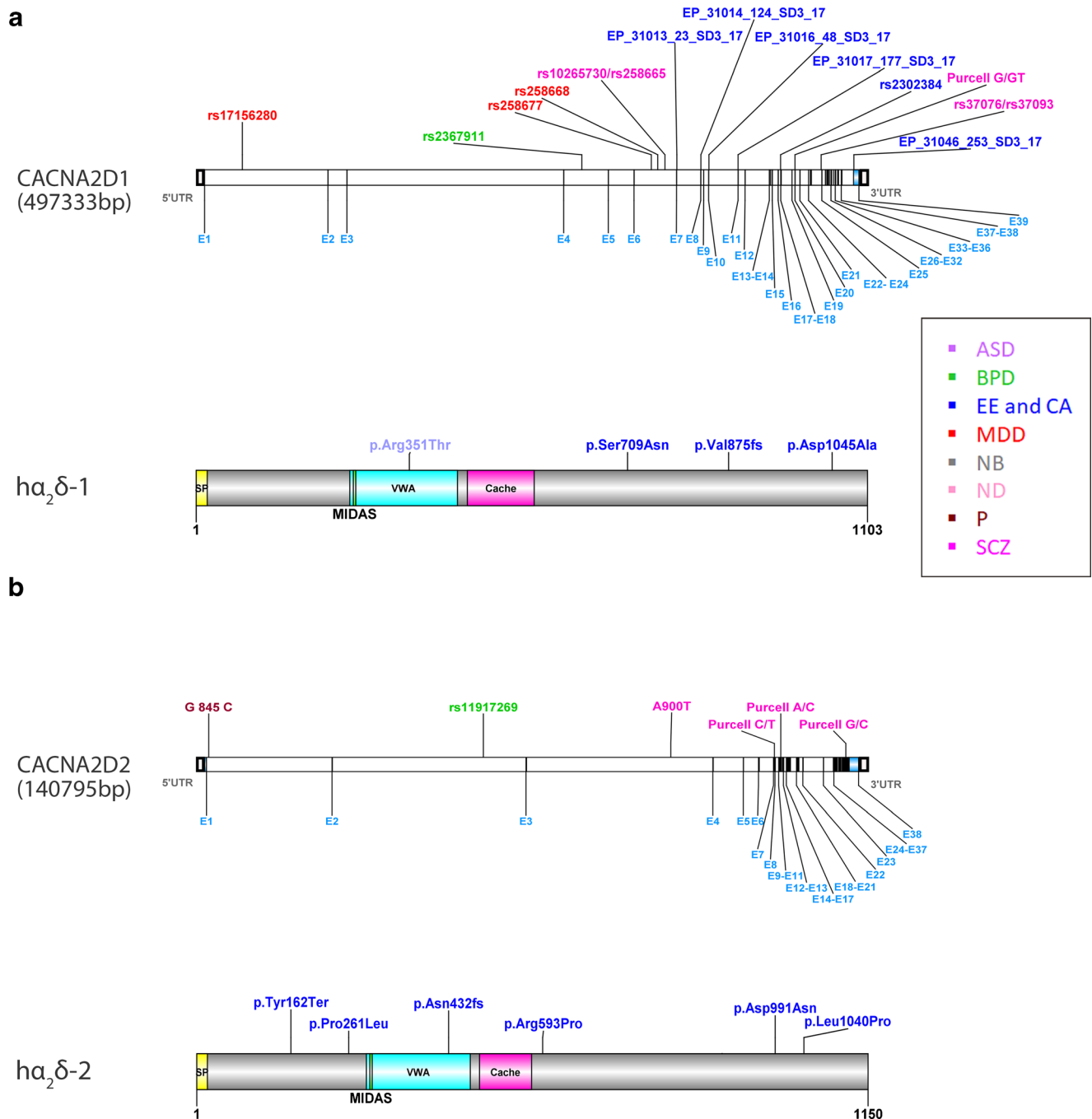
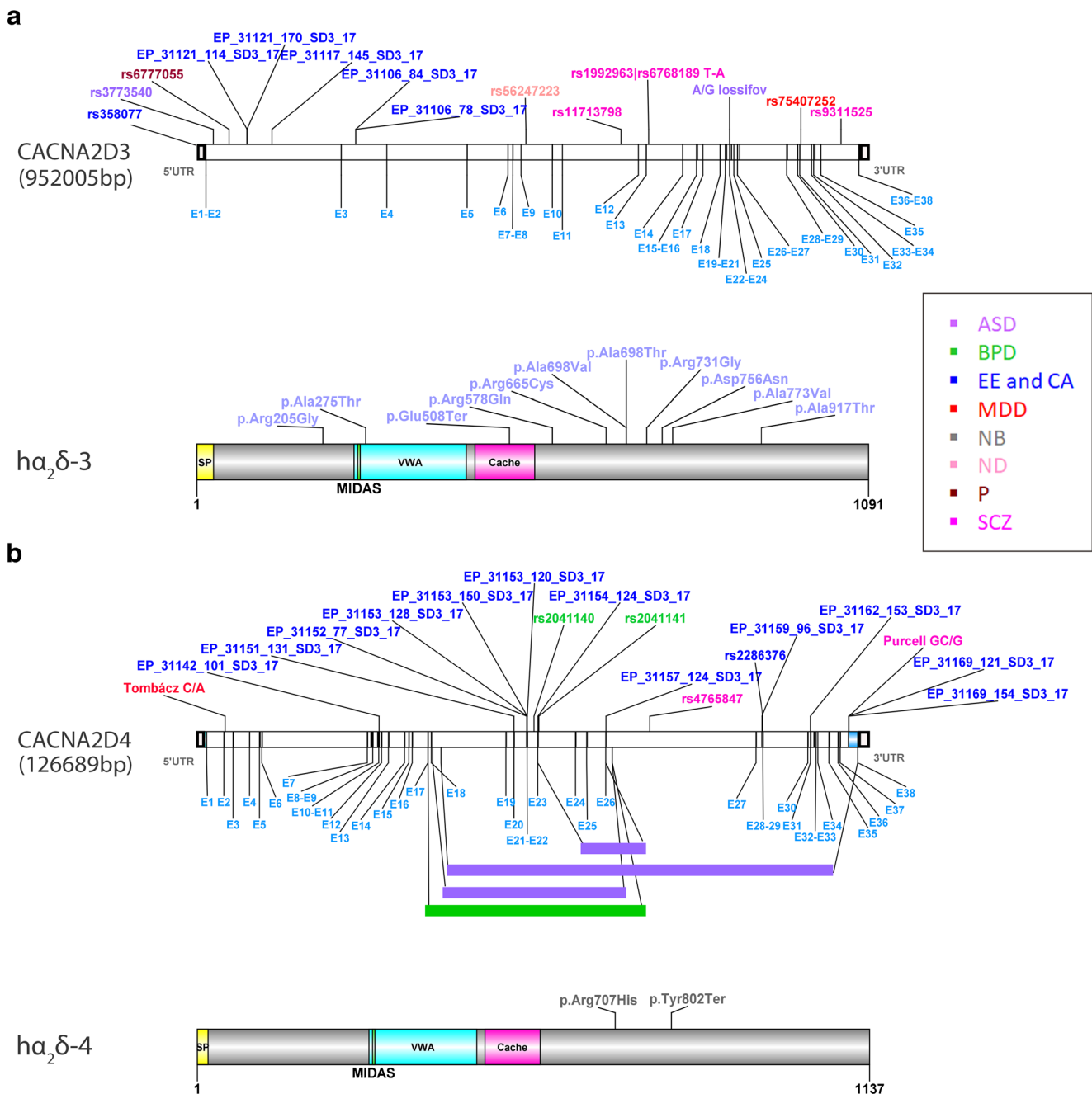


Fig. 1 Reference maps of predicted human $\alpha_2\delta$ -1 and $\alpha_2\delta$ -2 neuronal disease mutations and SNPs. Models of CACNA2D1 and CACNA2D2 genes (upper panels in **a** and **b**) and protein structures (lower panels in **a** and **b**), including exon positions (light blue) and protein domains, are based on Ensembl and UniProt databases (ENST00000356253.9/P54289; ENST00000266039.7/Q9N47). Previously published potentially disease-associated SNPs and disease mutations are indicated (see text for references). The protein structure of all $\alpha_2\delta$ proteins is highly

conserved sharing an N-terminal signal peptide (SP, yellow), a von Willebrand factor A domain (VWA, turquoise), a cache domain (magenta), and a MIDAS site (green) (see also Fig. 2). ASD (violet), autism spectrum disorders; BPD (green), bipolar disorder; CA (blue), cerebellar atrophy; EE (blue), epileptic encephalopathy; MDD (red), major depressive disorder; NB (gray), night blindness; ND (nude), nicotine dependence; P (bordeaux), pain; SCZ (magenta), schizophrenia

the $\alpha_2\delta$ -1 isoform complexed with the skeletal muscle calcium channel [126]. $\alpha_2\delta$ proteins undergo multiple posttranslational modifications, which make them rather unique, considering that proteolytic processing is a rare feature in VWA-containing

proteins. Generally, VWA domains by means of their metal ion-dependent adhesion site (MIDAS) are involved in protein-protein interactions such as extracellular matrix-cell adhesion proteins [124]. While all $\alpha_2\delta$ subunits contain a MIDAS motif,



only $\alpha_2\delta$ -1 and $\alpha_2\delta$ -2 incorporate the “perfect” MIDAS motif in which the presence of all five coordinating amino acids is predicted [14, 124]. This implies that structural rearrangement of the protein complex may occur upon divalent cation-dependent complex formation with a protein ligand [31]. Moreover, the

MIDAS site is involved in protein-protein interactions that are required to promote the anterograde transport of calcium channels [14, 48]. A perfectly conserved MIDAS motif is not required for metal ion binding [112]; however, it is not yet known whether differences in the MIDAS motif between $\alpha_2\delta$ subunits

might account for functional heterogeneity. A role of $\alpha_2\delta$ in extracellular protein interactions is further supported by the presence of cache domains, putative protein interaction sites with homology to the extracellular domains of bacterial chemosensing proteins [2, 31].

$\alpha_2\delta$ proteins as subunits of voltage-gated calcium channels

VGCCs are critical components of all types of excitable cells. They translate membrane depolarization into cellular functions like skeletal and cardiac muscle contraction, nerve cell signaling, hormone secretion, as well as the regulation of gene transcription. In the central nervous system, calcium channels are particularly involved in presynaptic neurotransmitter release and postsynaptic signaling. VGCCs are classified into three low-voltage-gated ($\text{Ca}_v3.1$ – 3.3) and seven high-voltage-gated ($\text{Ca}_v1.1$ – 1.4 , $\text{Ca}_v2.1$ – 2.3) channels. High-VGCCs constitute protein complexes classically consisting of a main pore-forming α_1 subunit and auxiliary intracellular β (β_{1-4}) and extracellular membrane-attached $\alpha_2\delta$ ($\alpha_2\delta$ -1 to $\alpha_2\delta$ -4) subunits. While the biophysical channel properties are defined by α_1 subunits, β subunits regulate membrane expression and other modulatory functions (reviewed in [11]). A wealth of heterologous co-expression studies has illustrated the roles of $\alpha_2\delta$ proteins as calcium channel subunits. Depending on the co-expressed α_1 subunit, $\alpha_2\delta$ subunits enhance current densities, modulate activation and inactivation kinetics, and can account for a hyperpolarizing shift in the voltage dependence of activation and inactivation (reviewed in [31, 56, 75]). The main proposed mechanism underlying the $\alpha_2\delta$ -induced increase in maximum current is an enhanced plasma membrane expression of channel complexes coupled with a decrease in their turnover [31]. Although it is still elusive how $\alpha_2\delta$ subunits implement their effects, the MIDAS motif seems to be essential, since a MIDAS mutant of $\alpha_2\delta$ -2 causes intracellular retention of α_1 subunits [14]. Due to their extracellular localization, $\alpha_2\delta$ subunits may also link extracellular signaling entities directly to the calcium channel complex.

Calcium channel-independent roles of $\alpha_2\delta$ proteins

An increasing number of recent studies suggests that individual $\alpha_2\delta$ isoforms exert specific neuronal functions beyond their classical role as calcium channel subunits. Hence, the affinity and strength of the interaction between α_1 and $\alpha_2\delta$ are under debate (see discussion in [75]). For example, a comprehensive quantitative study of the neuronal Ca_v2 channel proteome revealed molar ratios of 0.1–1% of $\alpha_2\delta$ compared to α_1 and β subunits [71], suggesting a relatively weak and possibly dynamic association. These findings are further corroborated by single-molecule tracking displaying that α_1 and $\alpha_2\delta$

are only transiently confined together at the cell surface of hippocampal neurons [10, 96, 119], and that $\alpha_2\delta$ -2 accumulation in lipid rafts may be partly independent from its interaction with presynaptic calcium channels [24]. Recent studies particularly implicate $\alpha_2\delta$ proteins as important regulators of synaptic functions and synapse formation, and these functions may be partly or entirely independent of the calcium channel complex (e.g. [18, 35, 40, 59, 97]).

$\alpha_2\delta$ proteins and neurological disorders

Dysfunctions of voltage-gated calcium channels have been linked to a variety of neurological disorders including Parkinson's disease, epilepsy, migraine, ataxia, neuropathic pain, and psychiatric disorders [3, 8, 17, 43, 80, 89, 105, 107, 132]. Hence, it is not surprising that likewise $\alpha_2\delta$ proteins have been associated with many of these diseases. The link to neurological disorders is further strengthened by the existence of a variety of knockout and mutant mouse models, which partly represent pathological features of identified human diseases (reviewed in [39]). In the last years, various genome-wide association studies (GWAS) as well as exome sequencing studies have contributed to identifying risk genes for neurological disorders and disease mutations. A further effort in detecting potential disease related genes has been made by single nucleotide variant (SNV) analysis and single nucleotide polymorphism (SNP) genotyping. Along these lines, copy number variation (CNV) analysis contributed largely to the understanding of disease predisposition. SNPs are point mutations of both coding and noncoding DNA regions that occur at a frequency of 0.5–1% in the population. SNV refers to a variation of a single nucleotide mostly arising in somatic cells. Both SNPs and SNVs can affect gene transcription or may generate splice sites. CNV is a form of variation, which affects the number of copies of a gene, resulting in increased or reduced protein expression. In the following sections, we give an overview of known and proposed $\alpha_2\delta$ disease associations. To this end, we distinguish between disease mutations in the proteins, which have been at least partially verified or discovered in specific patients and identified genetic disease associations for the human genes *CACNA2D1*–*4*, which partially lack experimental confirmation.

Disease mutations in $\alpha_2\delta$ proteins

Here, we briefly summarize human $\alpha_2\delta$ protein mutations, which were identified in PubMed, ClinVar, and SFARI Gene databases and which can be traced back to at least one patient. However, until today, the functional consequences of these mutations were either not or only partially characterized.

$\alpha_2\delta-1$

Missense or protein-disrupting mutations in $\alpha_2\delta-1$ have mainly been found in epileptic disorders (Fig. 1, $h\alpha_2\delta-1$). Two large genomic deletions affecting the genetic region of $\alpha_2\delta-1$, a de novo mutation (8.2 Mb deletion) and an inherited mutation (3.9 Mb deletion), were identified in patients with epileptic encephalopathy [68]. The same study identified additional inherited disease mutations (p.Ser709Asn and p.Asp1045Ala) occurring in two families: family 1 with an unaffected mother and dizygotic twin having the same mutation and family 2 with an unaffected father and affected monozygotic twin having the same mutation. Another de novo mutation (p.Val875fs) was discovered in a West syndrome patient [44]. A whole exome sequencing (WES) study revealed a de novo mutation p.Arg351Thr in a family of the Simons Simplex Collection (SSC) of patients with autism spectrum disorders (ASDs) [51].

$\alpha_2\delta-2$

Disease mutations in $\alpha_2\delta-2$ have been identified in epileptic encephalopathy and cerebellar ataxia patients (Fig. 1, $h\alpha_2\delta-2$). Edvardson et al. [34] described the p.Leu1040Pro mutation located in exon 36, close to the missense mutation p.Asp991Asn in exon 34 [115]. Another missense mutation (p.Pro261Leu) was identified in exon 7 [12]. Punetha et al. [84] found two families with epileptic encephalopathy: one displayed a missense variant mutation in exon 20 (p.Arg593Pro) while the other family exhibited a nonsense mutation in exon 5, predicting a severely truncated protein (p.Tyr162Ter). Furthermore, a CACNA2D2 variant (A900T) was found in schizophrenia (SCZ) patients within a Spanish population [92]. Finally, a frameshift mutation in exon 13 (p.Asn432fs) is likely causal in a family with epileptic encephalopathy [81].

$\alpha_2\delta-3$

Potential disease-causing mutations in $\alpha_2\delta-3$ were identified in patients with ASDs (Fig. 2, $h\alpha_2\delta-3$). Exome sequencing identified several synaptic, transcriptional, and chromatin genes disrupted in ASD including the $\alpha_2\delta-3$ mutations p.Arg578Gln, p.Arg665Cys, p.Ala698Thr, p.Arg731Gly, p.Asp756Asn, p.Arg205Gly, and p.Glu508Ter [28]. $\alpha_2\delta-3$ de novo gene mutations were also found in a Chinese ASD cohort (p.Ala773Val and p.Ala275Thr) [121] and in probands from the autism clinical and genetic resources in China (p.Ala917Thr and p.Ala698Val) [42].

$\alpha_2\delta-4$

$\alpha_2\delta-4$ mutations are strongly involved in retinal disease and gliomas, which go beyond the scope of the current review. However, we would like to highlight two prominent mutations (Fig. 2, $h\alpha_2\delta-4$), which affect $\alpha_2\delta-4$ expression or structure and may, in theory, also affect the previously suggested expression of $\alpha_2\delta-4$ in the brain [95, 117]. A mutation found in night blindness (p.Tyr802Ter) results in a premature stop at amino acid 802. However, it is expected that the mutated messenger RNA (mRNA) is recognized by the nonsense-mediated decay machinery [127]. Another mutation that deserves mentioning is p.Arg707His found to be involved in cone-rod dystrophy [4]. In addition, large genomic deletions affect $\alpha_2\delta-4$ protein expression and thereby cause retinal degeneration and night blindness. So far, no brain-related phenotypes were reported, although the gene has been identified as a risk gene for psychiatric disorders. One deletion of 35,740 bp comprising amino acids 574–850 was found in patients with bipolar disorder (BPD) [116].

Genetic disease associations

In this chapter, we briefly summarize human CACNA2D disease associations, which were identified in PubMed, ClinVar, and dbSNP databases. Mutations and SNPs that were not validated in separate publications were not included.

CACNA2D1

Over the past years, various genetic studies implied CACNA2D1, the gene encoding the $\alpha_2\delta-1$ isoform, in various forms of epilepsy and psychiatric disorders including major depressive disorders (MDDs), BPD, and SCZ (Fig. 1, CADNA2D1). In GWAS, CACNA2D1 was identified as a potential drug target in MDD [49] and SNP rs17156280 was associated with an interaction between depressive states and stressful events [50]. Furthermore, two SNPs (rs258668 and rs258677) were linked to depressive traits such as subjective well-being and neuroticism [76]. GWAS analysis of data from the Bipolar Disorder Genome Study Consortium [125] identified SNP rs2367911 as a risk for BPD with comorbid binge eating. In a haplotype analysis on Han Chinese population, two SNPs (rs37076|rs37093 G-C and rs10265730|rs258665 G-A) were associated with SCZ [134]. Within a Swedish population, a disruptive (frameshift) variant in CACNA2D1 has been identified in an exome sequence of schizophrenia patients [85]. A large deletion of CACNA2D1 was found in one SCZ patient in a Japanese population [66]. By comparing epileptic patients with control subjects, 23 SNPs were identified, of which 6 occurred exclusively in affected individuals [55]. From these 6 SNPs, one was located in the coding sequence (exon 11), one in the 3' UTR, while the other 4 SNPs

were located in introns including SNP (rs2302384), which was already known from the dbSNP database. Importantly, chromosomal deletions affecting CACNA2D1 were identified in three patients with epilepsy and intellectual disability [118].

CACNA2D2

Three *de novo* variations in CACNA2D2 (Fig. 1, CACNA2D2) were found in patients with SCZ [85]. Two out of three CACNA2D2 variations introduced premature stop codons while the third variant was predicted to disrupt a splice donor site (Purcell A/C). Another CACNA2D2 SNP (rs11917269) has been associated with BPD [103].

CACNA2D3

Initially, the gene encoding for $\alpha_2\delta$ -3 was characterized as a target for pain treatment, since the SNP rs6777055 was associated with reduced thermal pain response [72]. Nevertheless, more recently, CACNA2D3 has been linked to neurodevelopmental disorders such as SCZ, BPD, MDD, nicotine dependence (ND), and especially to ASD [52] (Fig. 2, CACNA2D3). For example, the genetic region of CACNA2D3 (3p14) was associated with SCZ and an endophenotype related to the function of the temporal lobe, the anti-saccade reflex [63]. In another study, pathway analysis of SCZ risk SNPs suggested an association of CACNA2D3 with the response to lurasidone, an antipsychotic drug used to treat SCZ [62]. Furthermore, regional enrichment analysis associated the genomic region 3p21.1_1, which also contains CACNA2D3, with SCZ and BPD [63], a link which also is supported by other studies [63, 67, 70, 101, 134]. While the abovementioned SNPs are all located in exons, one intronic SNP (rs75407252) has been linked to MDD [33]. Associations with ASD were identified in whole genome sequencing studies as inherited variations resulting in splicing disruption [131] or as CNVs [41]. Moreover, SNP rs3773540 was among the top 15 SNPs contributing to ASD diagnosis [100] and a splice site mutation at the beginning of exon 24 was identified in an ASD patient [52]. In their exome sequencing study, De Rubeis et al. [28] further identified several inherited variations in CACNA2D3 with so far unknown effect. Finally, 30 CACNA2D3 SNPs were identified in a study on epilepsy, of which 6 occurred in epileptic patients [55].

CACNA2D4

$\alpha_2\delta$ -4 is almost exclusively expressed in the retina, pituitary gland, and adrenal gland [29, 88]. However, very low levels are also detected in the hippocampus and are upregulated during development and status epilepticus [95, 117]. A possible role in the brain, which is further outlined below, is suggested by several SNPs linked to numerous neurological disorders.

For instance, a SNP located between the CACNA2D4 and CACNA1C genes (rs1024582) was significantly associated with cross-disorders that included attention deficit hyperactivity disorder (ADHD), BPD, ASD, SCZ, and MDD [22]. A *de novo* frameshift mutation likely disrupting CACNA2D4 was found in patients with SCZ [85] (Fig. 2, CACNA2D4). Another endophenotype of SCZ is default mode network, which was found to associate with the SNP rs4765847 [67]. SNPs rs2041140 and rs2041141 were linked to BPD [83], and partial deletions of 35.7 kb, eliminating exons 17–26, were found in two unrelated patients with late-onset BPD, one deletion was found in control individuals [116]. A WES study, comparing brain samples from MDD patients that died from suicide with MDD patients dying from unrelated causes, found a splice donor variant (C/A) [111]. Furthermore, a rare homozygous deletion affecting CACNA1C and CACNA2D4 (12p13.33) was found in a male ASD patient [102]. Thirty-nine SNPs were associated with epilepsy, of which 13 were exclusively found in the patients and not in controls [55] (Fig. 2, CACNA2D4).

Potential disease mechanisms

The overall picture of identified disease mutations suggests the involvement of $\alpha_2\delta$ proteins in epilepsy (particularly $\alpha_2\delta$ -2 but also $\alpha_2\delta$ -1) and ASD (mainly $\alpha_2\delta$ -3 but also $\alpha_2\delta$ -1). The majority of identified mutations represent missense mutations resulting in single amino acid substitutions and, to a lesser extent, protein truncations resulting in the complete or partial loss of $\alpha_2\delta$ proteins (e.g. p.Tyr162Ter in $\alpha_2\delta$ -2 or p.Glu508Ter in $\alpha_2\delta$ -3). When considering genetic disease associations, the picture becomes more diverse and links $\alpha_2\delta$ genes additionally to MDD ($\alpha_2\delta$ -1, $\alpha_2\delta$ -2, and $\alpha_2\delta$ -3), SCZ ($\alpha_2\delta$ -1, $\alpha_2\delta$ -2, $\alpha_2\delta$ -3, and $\alpha_2\delta$ -4), BPD ($\alpha_2\delta$ -1, $\alpha_2\delta$ -2, and $\alpha_2\delta$ -4), and ND ($\alpha_2\delta$ -3) [129]. While some of the described SNPs are in exons and most likely result in missense mutations, many are found in intronic DNA stretches or are affecting splice sites. As $\alpha_2\delta$ proteins, on the one hand, are important modulators of calcium channels and, on the other hand, are independent regulators of synaptic functions, disease mutations can affect these functions either independently or concomitantly. In order to understand which cellular functions may be compromised by $\alpha_2\delta$ mutations, we will here recapitulate evidence for the involvement of $\alpha_2\delta$ proteins in calcium channel-dependent and calcium channel-independent functions.

Calcium channel-dependent mechanisms

In heterologous co-expression studies, all $\alpha_2\delta$ proteins show similar effects on channel surface expression (reviewed in [25, 39, 57]) (Fig. 3, point 1). In contrast, evidence in differentiated

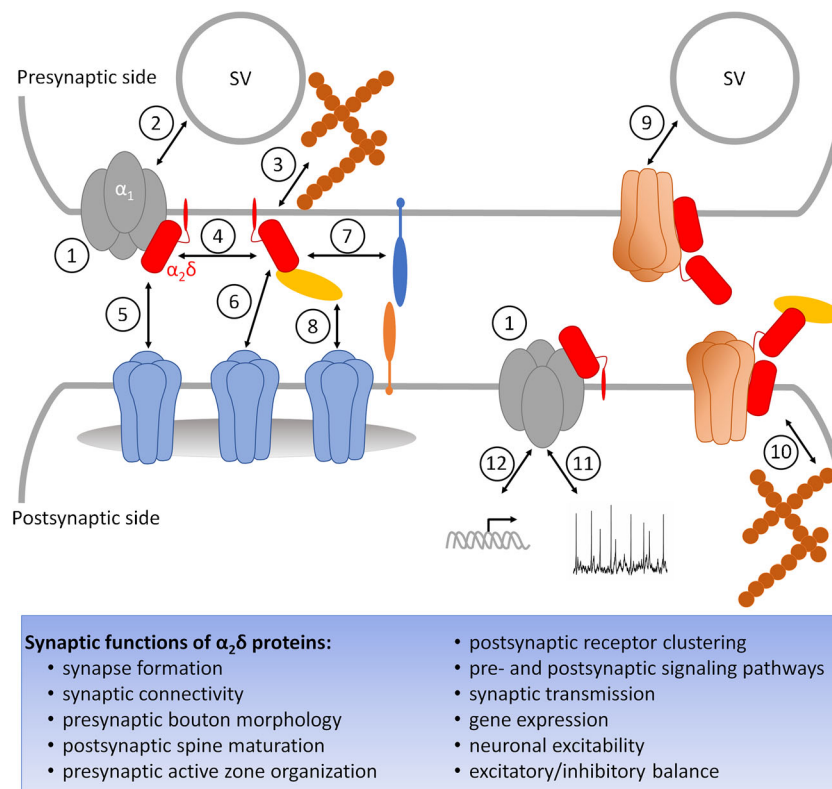


Fig. 3 Model summarizing proposed synaptic functions of $\alpha_2\delta$ proteins. $\alpha_2\delta$ proteins as calcium channel subunits enhance plasma membrane expression and modulate current properties of both presynaptic and postsynaptic α_1 subunits (1). At the presynaptic terminal, $\alpha_2\delta$ proteins mediate the accumulation of synaptic vesicles (SV, 2). They regulate active zone architecture (AZ) and bouton morphogenesis (3) either directly by interacting with proteins of the AZ and cytoskeleton (3) or indirectly via the VGCC complex (4). By aligning the presynaptic AZ with the postsynaptic membrane and postsynaptic AMPAR and GABA_AR (receptors in blue; 5 and 6, respectively), they act as trans-synaptic organizers either partly (5) or entirely (6) independent of the VGCC complex. This may be mediated by a direct interaction with

postsynaptic receptors (6), by interacting with prototypical cell adhesion molecules such as presynaptic neuexins and postsynaptic neuroligins (7), or by interacting with proteins of the extracellular matrix or secreted proteins (e.g. BDNF, TSP; yellow ellipses) (8). A transmembrane form of $\alpha_2\delta$ -1 bound to TSP is suggested to initiate the recruitment and stabilization of NMDAR (receptors in auburn) on the presynaptic (9) and postsynaptic (10) surface, a mechanism which contributes to enhanced synaptic transmission (9) and regulates intracellular signaling pathways as well as dendritic spine maturation via a small Rho GTPase (10). By regulating calcium currents of VGCC, $\alpha_2\delta$ proteins are further predicted to modulate neuronal excitability (11) and gene expression (12)

native cellular systems suggests at least a partial isoform specificity. In rat superior cervical ganglion neurons, for example, recombinant expression of $\alpha_2\delta$ -1 and $\alpha_2\delta$ -2 differently affected $Ca_v2.2$ expression when compared with $\alpha_2\delta$ -3 [98]. In mice, genetic ablation of $\alpha_2\delta$ -1 abolishes $Ca_v2.2$ cell surface expression in dorsal root ganglion (DRG) neurons and dramatically reduces dorsal horn expression [73]. Deletion of $\alpha_2\delta$ -3 differentially affects calcium currents; it reduces P/Q- and R-type currents, while N- and L-type remain unaltered [104]. Loss of $\alpha_2\delta$ -3 blocks homeostatic modulation of neurotransmitter release due to a failure to potentiate presynaptic calcium influx [122].

Kinetic and voltage-dependent properties of the channels are also modulated by $\alpha_2\delta$ proteins: depending on the channel type and the native channel environment, activation and inactivation kinetics can be slowed or accelerated [74, 75, 114, 120]. The voltage dependence of steady-state inactivation is generally hyperpolarized by $\alpha_2\delta$ [36],

while a shift to more positive potential is observed in individual cases [87]. In this context, it is noteworthy that the presence of $\alpha_2\delta$ -1 facilitates channel activation by increasing the voltage sensitivity of the channel's voltage-sensing domains [93]. Another important structural property of $\alpha_2\delta$ which affects calcium currents includes the VWA domain, as a MIDAS mutant of $\alpha_2\delta$ -2 subunit failed to enhance $Ca_v1.2$, $Ca_v2.1$, and $Ca_v2.2$ currents [14].

In neurons of the brain and the sensory nervous system, calcium channel function can also be influenced by altered or aberrant interaction of $\alpha_2\delta$ proteins with other identified interaction partners. For example, thrombospondin (TSP)-4 can increase calcium currents after low-voltage depolarization and contributes to aberrant excitatory synaptogenesis associated with neuropathic pain development; both of these effects can be blocked by gabapentin [77, 130]. The synaptic cell-adhesion molecules α -neuexins together with $\alpha_2\delta$ -1 regulate presynaptic calcium influx through $Ca_v2.1$ channels [10]

(Fig. 3, points 1, 4, and 7). The inhibitory effect of prion protein [99] may be modulated by competing with $\alpha_2\delta$ for GPI-anchoring pathways [1]. Moreover, low-density lipoprotein receptor-related protein 1 (LRP1) reduces $\alpha_2\delta$ -1 trafficking and hence influences trafficking of $\text{Ca}_v2.2$ to the cell surface [53]. Another protein suggested to influence VGCC function by interacting with $\alpha_2\delta$ -1 is the α subunit (Slo1) of the large conductance calcium-activated potassium (BK) channel [133]. Following spinal nerve injury, Slo1 regulates excitability by preventing the association of $\alpha_2\delta$ -1 and α_1 subunits, resulting in reduced functional membrane expression of $\text{Ca}_v2.2$ and analgesia. Similar mechanisms to counteract neuronal hyper-excitability might also be relevant in pathologic conditions, for example in epilepsy.

It is worth mentioning that an $\alpha_2\delta$ -like protein, CACHD1, has recently been characterized [20]. CACHD1 causes a functional increase of low voltage-activated T-type (Ca_v3) currents, which are not modulated by classical $\alpha_2\delta$ proteins. However, despite having a disrupted MIDAS motif, rat *Cachd1* also can increase $\text{Ca}_v2.2$ currents and surface expression upon heterologous co-expression [23].

Taken together, it is well established that $\alpha_2\delta$ proteins have significant effects on the biophysical properties of calcium channels, particularly on calcium current density, activation and inactivation kinetics, and voltage dependence of activation and inactivation. Thus, potential mutations affecting $\alpha_2\delta$ expression levels, glycosylation, or attachment of the GPI anchor may generally affect calcium channel surface expression and hence calcium currents. The p.Leu1040Pro mutation associated with epilepsy [34] resulted in reduced current densities and slowed inactivation when co-expressed with neuronal calcium channels. Similarly, a point mutation in the VWA domain, identified in a patient with autism (p.Arg351Thr) [51], prevents stable plasma membrane expression of $\alpha_2\delta$ -1 in a heterologous expression system [90]. Both mutations suggest that these effects can be mediated via altered expression of presynaptic and postsynaptic calcium channels or defective calcium currents which may, ultimately, result in an aberrant excitatory-inhibitory balance and underlie the pathophysiology of the associated diseases (Fig. 3). Interestingly, structure-homology modeling revealed two critical interactions of Arg351 (data not shown); hence, a significant effect of the exchange to Thr on the protein structure is possible. However, it is important to note that for the majority of identified human $\alpha_2\delta$ mutations, the structure-function consequences have not yet been studied.

Channel-independent synaptic functions of potential relevance for neurological disorders

In this chapter, we summarize novel synaptic roles of $\alpha_2\delta$ proteins, which, alone or in combination with channel-dependent functions, may be casually involved in

neurological disorders: synapse formation, synaptic connectivity and postsynaptic receptor abundance, presynaptic architecture, and synaptic transmission (Fig. 3). The link between synaptic $\alpha_2\delta$ proteins and brain disorders is particularly relevant, as a common feature of neurological disorders is their linkage to synaptic dysfunctions, referred to as synaptopathies (reviewed in [113]). This particularly accounts for neurodevelopmental disorders such as ASD, intellectual disability (ID), Fragile X syndrome (FXS), Down syndrome, ADHD, epilepsy, neuropsychiatric disorders (e.g. BPD, SCZ), MDD, and neurodegenerative diseases such as Alzheimer's, Huntington's, and Parkinson's diseases. Correct assembly of glutamatergic and GABAergic synapses comprises a series of intricate steps, including the contact of presynaptic axons with specific cellular postsynaptic compartments of target cells, as well as the recruitment of presynaptic and postsynaptic proteins to form a functional synapse (reviewed in [21, 38, 61, 110]). Hence, aberrant expression of individual synaptic proteins as well as mutations affecting their function might cause perturbations in synapse physiology and morphology. This, in turn, could lead to synaptic dysfunction, an imbalance of synaptic excitation and inhibition (E/I imbalance), abnormalities in wiring of neuronal circuits, and finally, the development of a neurological disease.

$\alpha_2\delta$ -1 mediates excitatory synaptogenesis

The interaction of postsynaptic $\alpha_2\delta$ -1 and its astrocyte-secreted ligand TSP mediates excitatory synapse formation in retinal ganglion cells, and the study suggests that $\alpha_2\delta$ -1/TSP-induced synaptogenesis does require neither presence nor function of α_1 subunits [35]. A follow-up study [90] demonstrated that loss of $\alpha_2\delta$ -1 in a conditional knockout mouse strain [78] impairs excitatory synaptogenesis and spine morphology in the cortex (Fig. 3, point 10). Interestingly, a point mutation in the VWA domain of $\alpha_2\delta$ -1, which has been linked to autism in humans [51], prevented both membrane trafficking of $\alpha_2\delta$ -1 (see above) and the rescue of the synaptogenic defects. These findings suggest that the previously identified $\alpha_2\delta$ -1 disease mutation might underlie impaired excitatory synapse formation and synaptic transmission. The study by Risher et al. [90] also proposes that TSP binding to a transmembrane form of $\alpha_2\delta$ -1 initiates the recruitment and stabilization of NMDA receptors (NMDARs) on the postsynaptic surface, a mechanism which may consequently contribute to dendritic spine maturation (Fig. 3, point 10). The idea that $\alpha_2\delta$ -1 may be also associated with presynaptic function is further suggested by studies addressing its involvement in neuropathic pain [18], hyperalgesia [30], and cultured hippocampal neurons [7]. Increased $\alpha_2\delta$ -1 expression levels via lentiviral injection [18] or induced by opioid application [30] potentiated presynaptic NMDAR trafficking in spinal cord synapses (Fig. 3, point 9). Excitatory synaptic transmission

was enhanced, ultimately provoking pain hypersensitivity, which could be normalized with either gabapentin or an $\alpha_2\delta$ -1 interfering peptide. The causative interaction of $\alpha_2\delta$ -1 with a set of distinct NMDAR subtypes (GluN1, GluN2A, and GluN2B) is presumably specific, as neither $\alpha_2\delta$ -2 nor $\alpha_2\delta$ -3 co-immuno-precipitated with NMDARs in the spinal cord and HEK cells [18]. To unravel the NMDAR interaction site within the $\alpha_2\delta$ -1 sequence, a chimeric approach was used, substituting the C-terminus, α_2 , or δ peptide of $\alpha_2\delta$ -1 with that of $\alpha_2\delta$ -2 or $\alpha_2\delta$ -3. Surprisingly, the interaction domain was identified to be located after the C-terminal GPI anchor cleavage site. As earlier studies revealed that $\alpha_2\delta$ subunits have hardly any or no cytoplasmic domain [26], these data might corroborate the abovementioned findings that NMDARs bind to a transmembrane form of $\alpha_2\delta$ -1 (for further detail and discussion, see [32, 46]) (Fig. 3, points 9 and 10). Both the hypothalamus [64, 65] and striatum [136] express presynaptic and postsynaptic $\alpha_2\delta$ -1/NMDAR complexes, augmenting NMDAR-mediated synaptic glutamate release in disease (hypertension) [64, 65] and under physiological conditions (cortico-striatal long-term potentiation in learning and memory) [136]. However, since a proteomic study of native rat brain [79] could not identify $\alpha_2\delta$ subunits in NMDAR-rich postsynaptic densities, it is conceivable that NMDAR/ $\alpha_2\delta$ -1 association might represent a rather dynamic form of neuronal signaling primarily relevant in neuropathological conditions.

It has been recently suggested that brain-derived neurotrophic factor (BDNF) might be an upstream regulator of neuronal $\alpha_2\delta$ -1 expression levels [9, 19, 86]. Lack of BDNF in mice causes reduced $\alpha_2\delta$ -1 cell surface expression in the ventromedial hypothalamus, likely affecting excitatory synapse formation without obviously altering calcium currents [19]. Moreover, BDNF mutant mice recovering from stroke display increased abundance of $\alpha_2\delta$ -1 and TSP2 together with a concomitant enhancement of glutamatergic synapses within the cortico-striatal pathway [86]. Importantly, as the study further identified a reduction of GABAergic innervation in distinct cortical layers, these data might suggest that synaptic $\alpha_2\delta$ -1 expression levels regulate the balance of excitation to inhibition.

$\alpha_2\delta$ -2 splice variants regulate synaptic connectivity and postsynaptic receptor abundance

Recent evidence suggests that presynaptic $\alpha_2\delta$ -2 potentially regulates synaptic connectivity and postsynaptic receptor abundance at specific central synapses. For instance, we previously found that solely $\alpha_2\delta$ splice variants lacking exon 23 regulate both the wiring of presynaptic axons to GABAergic postsynaptic sites, as well as postsynaptic GABA_A receptor (GABA_AR) abundance [40] (Fig. 3, points 5 and 6). As heterologous co-expression of distinct $\alpha_2\delta$ -2 splice variants with

various α_1 subunits caused similar effects on calcium current densities and activation/inactivation kinetics [45], these data suggest that the trans-synaptic function of $\alpha_2\delta$ -2 variants lacking exon 23 is independent of their role as a calcium channel subunit. Interestingly, $\alpha_2\delta$ -2 is also necessary for the proper spatial alignment of presynaptic L-type calcium channels and postsynaptic AMPA receptors in hair cell synapses of the inner ear [37], as well as for the structure and function of cerebellar climbing fiber synapses [5]. While the distinct signaling pathways remain to be determined, it is thus tempting to speculate that glutamatergic and GABAergic synapses may express a specific set of presynaptic $\alpha_2\delta$ isoforms and even splice variants in order to regulate synaptic connectivity and postsynaptic receptor abundance during development (Fig. 3, points 5 and 6). Along these lines, previous studies have proposed developmental functions of an $\alpha_2\delta$ -2 variant lacking exon 23 in establishing neuronal circuits [106, 108, 109]. As murine axonal projections of DRG neurons mature during embryonic development, they undergo a switch from a growth-competent (electrically dormant) to a transmitting (electrically active) phase, which also correlates with increased $\alpha_2\delta$ -2 expression levels [108]. Whether $\alpha_2\delta$ -2 regulates axon growth of sensory neurons by altering α_1 subunit-mediated neurotransmission needs to be clarified. However, recent findings in mouse cortical neurons show that $\alpha_2\delta$ -2, which is specifically expressed at the soma, axons, and growth cones of corticospinal layer V neurons, displays distinct postnatal expression patterns [106]. Therefore, increased $\alpha_2\delta$ -2 expression levels during development accompany increased spontaneous firing at a time point when cortico-spinal axon growth is nearly completed, and synaptogenesis begins.

Altogether, several lines of evidence suggest the existence of distinct spatiotemporal expression patterns of $\alpha_2\delta$ -2 regulating synapse connectivity and specificity, which may even depend on exon usage [40]. Since the amount of $\alpha_2\delta$ -2 protein is also increased in pathological conditions such as spinal cord injury [106, 108], abnormal expression levels likely contribute to maladaptive synaptogenesis or plasticity, ultimately leading to aberrant neuronal networks.

$\alpha_2\delta$ -3 regulates size, morphology, and architecture of presynaptic boutons

Most of today's knowledge on the role of $\alpha_2\delta$ -3 in synaptic transmission and synapse formation is based on studies conducted in invertebrate model systems. Caylor et al. [16] showed, for instance, that in *Caenorhabditis elegans*, homologs of Ca_v2 (UNC-2), $\alpha_2\delta$ (UNC-36), and CaMKII (UNC-43) regulate the size and morphology of GABAergic motoneuron terminals in neuromuscular junctions (NMJs) (Fig. 3, point 3). In *Drosophila melanogaster* null mutants of the $\alpha_2\delta$ -3 homolog straightjacket (stj), motoneuron terminals of NMJs fail to develop presynaptic boutons showing a severely

disrupted cytoskeleton [59]. Nevertheless, growth cones successfully navigate and halt at their target muscles, indicating that initial contact formation of synapses is normal but followed by an arrest of morphogenesis during larval development. Although the synapse-stabilizing protein ankyrin-2 is absent, functional presynaptic specializations are present and properly opposed to postsynaptic clusters of glutamate receptors. The Ca_v2 homolog cacophony, however, is missing in active zones, consistent with an inability to evoke synaptic release. Both bouton formation and function could be rescued when expressing an $\alpha_2\delta-3$ mutant lacking the δ peptide, suggesting that the synaptogenic function and calcium channel targeting property require the extracellular α_2 part of the protein. Importantly, the authors demonstrated that bouton formation does not depend on synaptic calcium channel localization, as the α_1 mutant cacophony displays normal morphogenesis. A recent study might offer a mechanistic explanation how the extracellular α_2 peptide of $\alpha_2\delta-3$ regulates the development of embryonic *Drosophila* NMJs. Hoover et al. [47] uncovered that presynaptic $\alpha_2\delta-3$ promotes the function of an activity-dependent autocrine bone morphogenetic protein (BMP) signaling pathway via modulating membrane retention of glass bottom boat (Gbb). Presynaptic Gbb, in turn, serves as a retrograde cue regulating active zone architecture, synaptic vesicle distribution, neurotransmitter release, and bouton morphogenesis. Similar mechanisms might also exist in vertebrate synapse formation, as altered expression of mammalian $\alpha_2\delta-3$ has been shown to affect the size and morphology of presynaptic boutons of auditory nerve fibers [82] (Fig. 3, point 3), and GABAergic synapses of central neurons as well as postsynaptic receptor abundance [40] (Fig. 3, points 5 and 6). In hippocampal neurons, overexpression of $\alpha_2\delta-3$, in contrast to $\alpha_2\delta-1$, facilitated spontaneous GABA release and increased the density of inhibitory synapses [7]. It is important to note that presynaptic $\alpha_2\delta-3$ and $\alpha_2\delta-2$ may regulate synaptic differentiation and postsynaptic receptor abundance by two independent mechanisms: while overexpression of $\alpha_2\delta-3$ induced smaller synapses, associated with reduced content of both presynaptic and postsynaptic proteins, presynaptic $\alpha_2\delta-2$ modulated postsynaptic GABA_A R abundance without affecting presynaptic bouton size [40].

$\alpha_2\delta-4$ is required for synaptic transmission and wiring of photoreceptors

$\alpha_2\delta-4$ transcript levels by far exceed those of other $\alpha_2\delta$ isoforms in retinal photoreceptor cells [58]. Accordingly, mutations in the human, mouse, and zebrafish genes [54, 94, 123, 127, 128] and the associated pathological phenotypes underline a general importance of $\alpha_2\delta-4$ in maintaining proper rod and cone synaptogenesis and physiology. Because $\alpha_2\delta-4$ regulates both functional membrane expression of $\text{Ca}_v1.4$ channels and synaptic transmission of rods

[123] and cones [54, 94], converging lines of evidence suggest that abnormal $\text{Ca}_v1.4$ expression may be the main cause for synaptic abnormalities of retinal photoreceptor cells. Nevertheless, these studies also implicate the presence of distinct $\alpha_2\delta-4$ signaling pathways in rod and cone photoreceptors. For instance, Wang et al. [123] showed that in $\alpha_2\delta-4$ knockout mice, the key synaptogenic molecule for rod synaptogenesis (Elfn1) is not recruited to rod synaptic terminals. This prevents rods from establishing contacts with their postsynaptic targets, the ON rod bipolar cells, which disrupt postsynaptic metabotropic glutamate receptor 6 (mGLUR6) clustering. Even though only little structural effects have been reported for cone photoreceptor synapses in this study, synaptic transmission appears to be severely impaired also in cones. A second report using a different $\alpha_2\delta-4$ knockout mouse [54] could further extend these findings in revealing that abnormal wiring of cone synapses is associated with impaired cone transmission through ON and OFF bipolar pathways. Nevertheless, abnormalities of ribbon synapses are more severe and $\text{Ca}_v1.4$ channels are lost faster in terminals of rods than in cones. Because Elfn1 is not expressed in cone synapses [15], the findings described in both reports [54, 123] indicate that loss of $\text{Ca}_v1.4$ may be the primary cause for synaptic abnormalities in rods and cones. Alternatively, it is conceivable that $\alpha_2\delta-4$ regulates rod and cone synapse structure by two distinct mechanisms (one dependent on Elfn1 and one independent from Elfn1). A recent study conducted in zebrafish underpinned a functional divergence and different developmental expression patterns of two $\alpha_2\delta-4$ variants in cone photoreceptors [94]. Interestingly, solely the loss of one variant, *Cacna2d4b*, specifically leads to the occurrence of mislocalized synapses during larval development. Thus, it is tempting to speculate that spatiotemporal expression patterns of distinct $\alpha_2\delta-4$ variants might regulate synapse connectivity and specificity of rod photoreceptors.

Until recently, protein and mRNA expression levels of human and murine $\alpha_2\delta-4$ seemed negligible in all previously examined CNS regions [40, 88, 95]. A recent study, however, reported increased levels of $\alpha_2\delta-4$ mRNA in human hippocampal biopsies obtained from epileptic patients, an interesting finding in spite of the lack of information on absolute mRNA levels [117]. Considering that standard curve-based qRT-PCR studies from our group identified very low amounts of $\alpha_2\delta-4$ mRNA in the mouse brain [40, 95], the recent findings suggest the existence of a subpopulation of hippocampal neurons expressing $\alpha_2\delta-4$. Interestingly, several studies provide a potential link between *CACNA2D4* and psychiatric disorders ([116], see discussion therein and above). Yet, it remains to be addressed whether late-onset bipolar disorder might be a secondary effect caused by visual impairments in these patients, or if $\alpha_2\delta-4$ might play a more prominent role in central neurons of the brain.

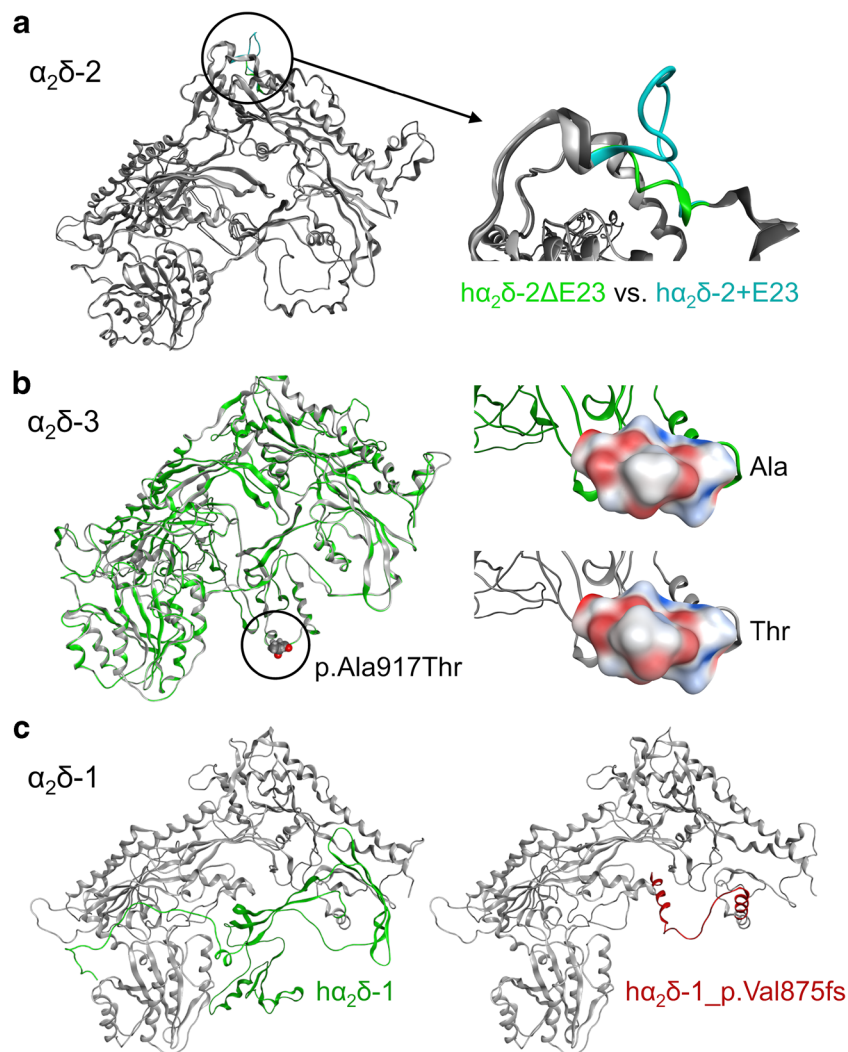


Fig. 4 Putative consequences of $\alpha_2\delta$ splicing and selected disease mutations on protein structure. Using homology modeling based on the 2.7 Å resolution structure of $\alpha_2\delta$ -1 (PDB code: 6JP8) [6, 135], we tested the potential consequences of alternative splicing of exon 23 on the structure prediction of $h\alpha_2\delta$ -2 (a) [40], the potentially autism-causing mutation p.Ala917Thr in $h\alpha_2\delta$ -3 (b) [42], and the $h\alpha_2\delta$ -1 frameshift mutation p.Val875fs associated with epilepsy (c) [44]. **a** As previously suggested for mouse $\alpha_2\delta$ -2 [40], the inclusion of exon 23 in $h\alpha_2\delta$ -2 suggests the formation of an extra loop leading to the disruption of an α -helix present. **b** Mutation p.Ala917Thr is not predicted to alter $h\alpha_2\delta$ -3

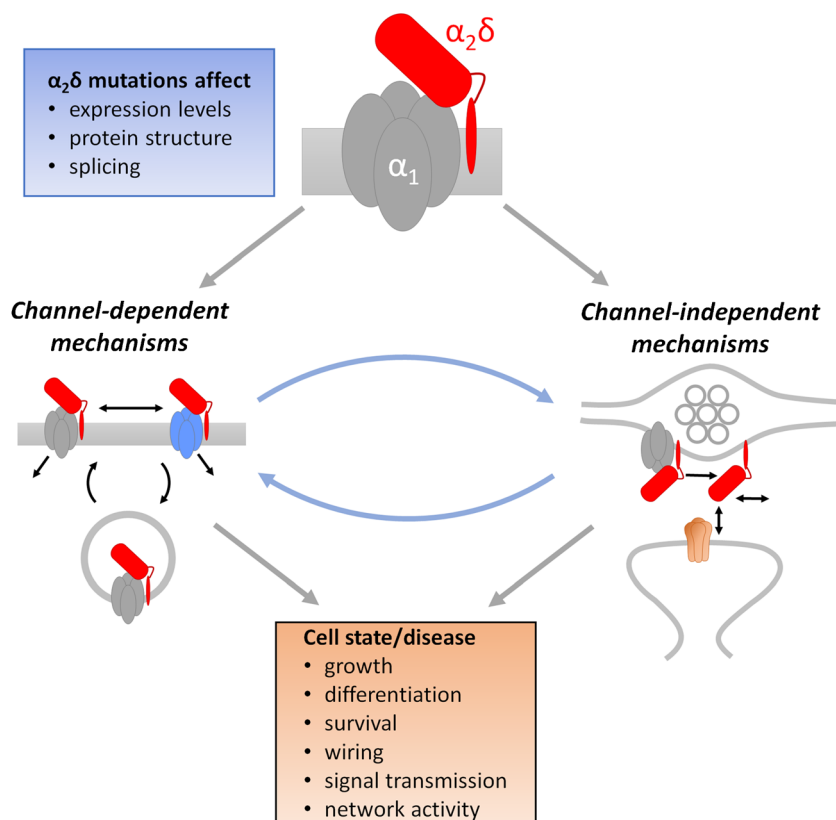
protein structure; however, altered electrostatic potential (EP) on the surface (red, negative EP; blue, positive EP) and hence altered surface hydrophobicity may influence protein interactions. **c** The $h\alpha_2\delta$ -1 frameshift mutation p.Val875fs deletes the C-terminal part of α_2 and the entire δ peptide (green). Furthermore, the altered reading frame introduces a random 36-amino acid sequence stretch (red). Molecular modeling was performed with the MOE software [69]. EP calculations were performed using Amber 10:EHT charges and the Poisson-Boltzmann approach as implemented in the software. Prior to the calculations, the structure was prepared and protonated with Protonate3D within MOE

How do mutations affect $\alpha_2\delta$ protein function?

Considering the manifold roles involving $\alpha_2\delta$ proteins, it is obvious that $\alpha_2\delta$ malfunction can affect channel-dependent and channel-independent functions. Particularly, mutations affecting expression levels of $\alpha_2\delta$ proteins (e.g. intronic SNPs, protein truncations, CNV) may simultaneously affect both functions. Hence, the extent of the disease involvement will mainly depend on the regional and temporal expression of individual $\alpha_2\delta$ subunits. For example, this is the case in

epileptic encephalopathies and cerebellar ataxias associated with $\alpha_2\delta$ -2 (predominant expression of $\alpha_2\delta$ -2 in the cerebellum) or retinal dysfunctions associated with $\alpha_2\delta$ -4 (predominant expression of $\alpha_2\delta$ -4 in the retina). Similarly, mutations affecting splicing may primarily affect regions endogenously expressing specific splice isoforms. Although until today regional and temporal expression patterns of $\alpha_2\delta$ splice variants are still incompletely understood, splices are indeed relevant for the functional diversity [40, 60]. However, in contrast to mutations affecting $\alpha_2\delta$ protein expression levels, splicing

Fig. 5 Model summarizing channel-dependent and channel-independent roles of $\alpha_2\delta$ proteins. Mutations of $\alpha_2\delta$ genes can affect expression levels, protein structure, and splicing. These alterations have consequences on calcium channel–dependent functions (membrane expression, current modulation, channel subtype–specific functions), and channel-independent functions (extracellular and/or trans-synaptic interactions and signaling pathways). The two mechanisms should not be considered entirely independent as they are likely influencing each other. For example, alterations in synapse differentiation will also affect VGCC expression with further consequences on neuronal excitability and synaptic transmission



mutations may affect channel-dependent and channel-independent functions simultaneously or separately. Homology modeling based on an $\alpha_2\delta$ -1 cryo-EM structure [126, 135] revealed that inclusion of single spliced exon 23 in $\alpha_2\delta$ -2 resulted in the formation of an extra loop disrupting an α -helix [40] (Fig. 4a). Hence, inclusion of this exon prevents the trans-synaptic recruitment of postsynaptic GABA_A receptors. Since, based on the predicted structure, the region of the relevant exon is facing away from the channel into the synaptic cleft, it is conceivable that such structural alterations affect specific protein-protein interactions without modulating the calcium channel. Channel-independent structural interactions are supported by a very recent observation in our laboratory. While the integrity of the MIDAS motif in $\alpha_2\delta$ subunits was shown to be required for functional membrane expression of the channel complex [14, 48, 97], expression of MIDAS mutants could not rescue the accumulation of synaptic proteins in $\alpha_2\delta$ subunit triple knockout neurons [97].

The consequences of missense point mutations on channel-dependent and channel-independent functions are not that easily predictable without detailed experimental evidence. In theory, missense mutations may alter the entire structure of $\alpha_2\delta$ proteins. Moreover, single missense mutations may also affect the interaction of $\alpha_2\delta$ subunits with the channel itself or with extracellular or trans-synaptic interaction partners. Here, we used structure-homology modeling (see Fig. 4) to predict the consequences of previously identified potentially autism-

causing mutations in $\alpha_2\delta$ -1 and $\alpha_2\delta$ -3. While the p.Arg351Thr mutation may severely alter protein structure (see above), modeling of a specific mutation in $\alpha_2\delta$ -3 (p.Ala917Thr) [42] provides an indication that even subtle changes may alter specific interactions. The location of p.Ala917Thr in the predicted structure of $\alpha_2\delta$ -3 is a potential site for protein-protein interaction, partly facing towards the channel α_1 subunit and partly facing to the extracellular side (Fig. 4b). Homology modeling does not suggest strong effects of this amino acid substitution on the overall structure. However, slight alterations in the surface electrostatic potential and hence surface hydrophobicity may suffice to influence the stability with specific inter-channel-complex interactions or components of the extracellular matrix. Considering ASD, one may speculate that an altered surface structure may influence the weak and dynamic interaction with synaptic cell adhesion molecules, for example neurexins [10], thereby affecting both channel and synaptic functions. We also modeled the potential consequences of an $\alpha_2\delta$ -1 frameshift mutation likely causing epilepsy (p.Val875fs) [44]. While it is possible that altered mRNA may already trigger nonsense-mediated mRNA decay, an actually translated $\alpha_2\delta$ -1_p.Val875fs protein may be functionally affected in two ways: first, the lack of the δ peptide and membrane anchoring will affect the stability of its membrane localization and hence channel interaction, and second, the frameshift not only deletes a large portion of the C-terminal end of the protein including the entire δ peptide

but also introduces a sequence stretch of 36 amino acids which will likely interfere with potential remaining protein-protein interactions (Fig. 4c). Hence and considering that the p.Val875fs mutation was found in epileptic patients, it seems consistent with the hypothesis that its pathophysiology may be mediated partly by channel-dependent functions influencing neuronal excitability and/or synaptic transmission.

Future perspectives

Over the last couple of years, the number of disease associations of $\alpha_2\delta$ genes and proteins has been steadily increasing. The multitude of distinct neurological and neuropsychiatric disorders likely involving $\alpha_2\delta$ proteins supports their important roles in neuronal functions (Fig. 5). Hence, on top of the existing drugs gabapentin and pregabalin, $\alpha_2\delta$ proteins may provide highly specific targets for future and novel paradigms in treating neurological disorders. This seems particularly promising when calcium channel-dependent as well as calcium channel-independent functions could be separately targeted. However, until then, several basic immanent research questions need to be further elucidated: first, although recent studies identified $\alpha_2\delta$ isoform-specific signaling pathways, the role of functional redundancy between the different isoforms is not yet understood. For example, while specific $\alpha_2\delta$ isoforms are clearly associated with distinct synaptic functions (see above), synapse deficiency in a presynaptic triple knockout phenotype can be rescued by the expression of each individual $\alpha_2\delta$ protein [97]. Second, the role of individual $\alpha_2\delta$ isoforms in neuron-type- and synapse-type-specific signaling mechanisms needs to be resolved. In the brain, $\alpha_2\delta$ proteins show an isoform-specific distribution pattern; however, this pattern shows considerable overlap and at least three isoforms can be simultaneously expressed in a single neuron. Hence, understanding neuron- and synapse-type specificity in $\alpha_2\delta$ protein functions will become increasingly important. For example, the extremely low abundance of $\alpha_2\delta$ -4 in the brain may suggest the specific expression of this isoform in a lowly abundant neuronal cell type. Third, the definitive distinction between calcium channel-dependent and calcium channel-independent functions is until today an experimental challenge. Several recent studies identified $\alpha_2\delta$ -specific signaling pathways and protein-protein interactions (e.g. BK channel and NMDAR). Yet, in neurons, multiple calcium channel types are ubiquitously expressed and critically involved in basic signaling functions. Consequentially, malfunctioning $\alpha_2\delta$ proteins are likely to also affect calcium channel functions. Fourth, $\alpha_2\delta$ proteins are emerging as novel and critical trans-synaptic organizing molecules, which may provide a missing link in understanding synapse formation and differentiation. It will be critical to identify their specific trans-synaptic mode of action including potential extracellular or presynaptic and postsynaptic interaction partners. Finally, cryo-EM analysis provided first insights

into the structural organization of $\alpha_2\delta$ subunits, but until today, our understanding of neuronal $\alpha_2\delta$ proteins depends on structure-homology modeling based on the skeletal muscle calcium channel complex. In neurons, the actual structural organization of $\alpha_2\delta$ proteins may depend on their specific calcium channel associations and their potential extracellular and trans-synaptic interactions.

Taking together, $\alpha_2\delta$ proteins are critical neuronal signaling proteins involved in a variety of cellular and synaptic mechanisms and altered function may cause or mediate neurological disorders. Future research efforts in studying their specific neuronal roles will help in understanding the pathophysiology of associated disorders and may open the way for the development of novel therapeutic paradigms.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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