Calcium channel auxiliary $\alpha_2\delta$ and β subunits: trafficking and one step beyond

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Abstract | The voltage-gated calcium channel $\alpha_2\delta$ and β subunits are traditionally considered to be auxiliary subunits that enhance channel trafficking, increase the expression of functional calcium channels at the plasma membrane and influence the channels' biophysical properties. Accumulating evidence indicates that these subunits may also have roles in the nervous system that are not directly linked to calcium channel function. For example, β subunits may act as transcriptional regulators, and certain $\alpha_2\delta$ subunits may function in synaptogenesis. The aim of this Review is to examine both the classic and novel roles for these auxiliary subunits in voltage-gated calcium channel function and beyond.

Auxiliary subunits
In the context of ion channels, an auxiliary or accessory subunit does not have a direct role in forming the channel pore, but modifies the channels, affecting their trafficking, biophysical properties or pharmacology.

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In excitable cells, including neurons, voltage-gated calcium channels (VGCCs; also known as Ca_Vs) respond to a depolarization of membrane potential by allowing Ca^{2+} entry. This response provides Ca^{2+} for many processes, including neurotransmitter and hormone release, as well as calcium-dependent gene transcription. VGCCs also influence tonic activity in both central and peripheral neurons, and other cell types[1,2] (FIG. 1). The distinctive properties of the various subtypes of channel-forming subunits, and their modulation by auxiliary subunits, allow the function of VGCCs to be tailored to the various roles that they perform in different cell types and in different subcellular locations.

The functional diversity that is achieved by mammalian VGCCs is due to the existence of ten channel-forming α_1 subunits[3], as well as four $\alpha_2\delta$ and four β auxiliary subunits. There are also ten members of a γ subunit family, but whether γ subunits form part of neuronal calcium channels remains controversial[4–6], and they will not be discussed extensively in this Review. The α_1 subunits principally determine the kinetics and voltage dependence of VGCCs, as well as their pharmacology; however, these properties can be modulated by auxiliary $\alpha_2\delta$ and β subunits, which also have major roles in VGCC trafficking. Recent studies have led to a greater understanding of how these auxiliary subunits mediate their concerted trafficking roles, as well as revealing their actual or potential involvement in disease and in therapeutic interventions. It has also become evident that both the $\alpha_2\delta$ and β proteins may participate in processes that are separate from their roles as calcium channel subunits. This Review will first focus on the traditional functions of $\alpha_2\delta$ and β proteins as calcium channel subunits, and then outline their potential roles in various other neuronal processes.

Neuronal and muscle VGCCs

Calcium conductances were first noted in studies of invertebrate muscle and have now been identified in all types of excitable cell (for a review, see REF. 2). The voltage-gated calcium currents underlying these conductances were shown to have distinct high-voltage-activated (HVA) and low-voltage-activated (LVA) components in neurons. Following the development of calcium channel blockers, distinct subtypes of VGCC were identified, including the L-type calcium channels, which are sensitive to blockade by the 1,4-dihydropyridines (DHPs) and are usually activated at high voltages. Further classes of VGCC that can be defined according to their physiological and pharmacological characteristics are the P/Q-, N- and R-type HVA calcium channels, and the T-type LVA channels (for a review, see REF. 2). These subtypes are all present, in varying numbers, in neuronal tissue.

Subunit identification. VGCCs were first purified from membranes of skeletal-muscle transverse tubules. These membranes showed extensive binding to ^{3}H-DHPs, which were known to inhibit L-type VGCCs and were therefore used to identify the channels during the purification process[7]. SDS–PAGE showed that the purified skeletal muscle DHP receptor complex was composed of five bands, which were termed α_1 (~175 kDa), α_2 (~150 kDa), β (~54 kDa), δ (17–25 kDa) and γ (~32 kDa).

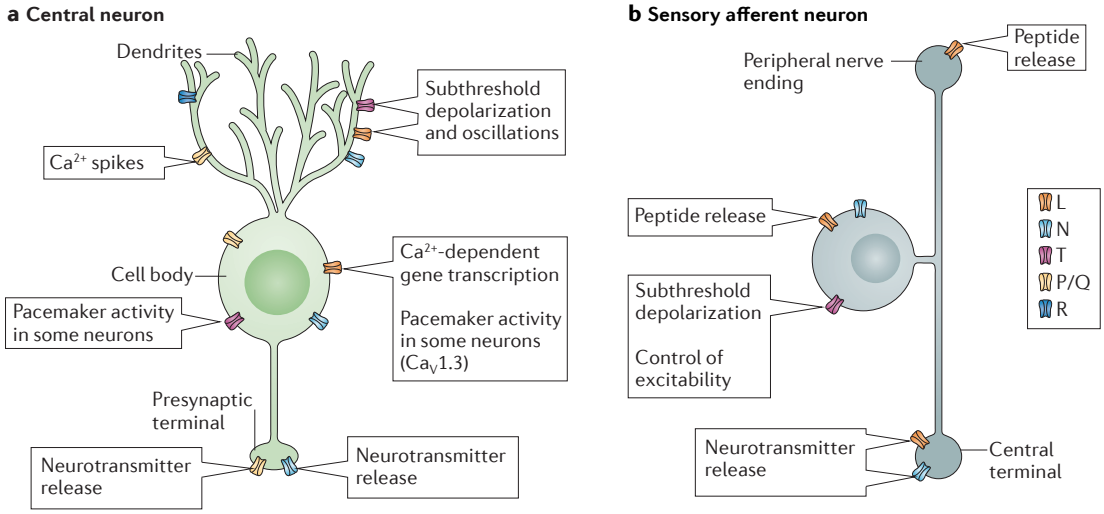


Figure 1 | **Distribution and roles of calcium channels in neurons. a** | In central neurons, dendritic and presynaptic terminal compartments have non-uniform distributions of the various voltage-gated calcium channels. The main distributions and functions of these channels are shown in the figure, but it should be noted that these vary widely among different classes of neuron. $Ca_V2.1$ (P/Q-type) and $Ca_V2.2$ (N-type) channels are present at presynaptic terminals, where they are intimately connected to the active zone and are required for neurotransmitter release. In many neurons, T-type channels are present in dendrites, where they participate in subthreshold oscillations and contribute to the regulation of firing patterns[17]. The medium voltage-activated $Ca_V1.3$ channels are also involved in oscillatory behaviour[166,167]. T-type channels may also have a presynaptic role in some neurons[168]. L-type channels participate in excitation–transcription coupling, providing the Ca^{2+} for Ca^{2+}-dependent gene transcription[169]. **b** | Pseudo-unipolar peripheral sensory neurons mainly use N-type channels for fast neurotransmitter release at their central terminals, whereas L-type channels are involved in peptide release from cell bodies and terminals. There is also evidence for calcium channels at peripheral terminals, contributing to peptide release. T-type channels also have a role in regulating excitability in some subtypes of sensory neuron. These neurons do not have dendrites.

The ^{3}H-DHPs were found to bind specifically to the α_1 protein. As DHPs were known to block calcium channels, it was asserted correctly that this protein was the pore-forming subunit (BOX 1). Notably, skeletal-muscle calcium channels co-purify with γ_1 subunits, but most evidence suggests that neuronal calcium channels do not associate with γ subunits[8]. However, these subunits do have effects on calcium channels in heterologous expression systems[4,9]. The main roles of the other members of the γ subunit family involve their interaction with AMPA-type glutamate receptors[6], although they are also involved in endosomal trafficking in neurons[10].

Following purification of the skeletal muscle α_1 subunit, a cDNA encoding this polypeptide was cloned[7]. Hydropathy analysis predicted that this subunit, termed α_1S, had 24 transmembrane α-helices, arranged as four homologous domains that are connected by intracellular loops, with amino and carboxyl termini (FIG. 2a). Nine other mammalian α_1 subunit cDNAs were subsequently cloned. Together with the α_1S subunit, the encoded subunits can be divided into three distinct subclasses, namely Ca_V1, Ca_V2 and Ca_V3 (FIG. 2b).

The Ca_V1 family has four members, all of which are sensitive to DHP agonists and antagonists, and are therefore termed L-type channels. $Ca_V1.1$ is the skeletal muscle isoform of this family, whereas $Ca_V1.2$ (α_1C subunit) predominates in cardiac muscle, although it is also present in neurons. $Ca_V1.3$ and $Ca_V1.4$ (α_1D and α_1F subunits, respectively) show a more restricted distribution than the other Ca_V1 family members, and are activated at lower voltage thresholds. $Ca_V1.3$ has important roles in neurotransmission in auditory hair cells and in cardiac pacemaker activity[11], whereas $Ca_V1.4$ has a predominant role in synaptic transmission in the retina[12,13]. The diverse tissue-specific roles of the Ca_V1 channels and their differential sensitivity to DHPs have been the subject of several recent reviews[14,15].

Members of the Ca_V2 channel class are insensitive to DHPs and have a mainly neuronal distribution. $Ca_V2.1$ (α_1A subunit) is the channel underlying the P/Q-type calcium currents identified physiologically, whereas $Ca_V2.2$ (α_1B subunit) is the counterpart of the neuronal N-type calcium channels. $Ca_V2.3$ (α_1E subunit) corresponds to the residual R-type calcium current, which can be detected when N-, P/Q- and L-type channels are inhibited. The Ca_V3 group of channels (α_1G, α_1H and α_1I subunits), which are present in many excitable cells, are the molecular counterparts of the T-type channels. This family of LVA channels shows greater sequence divergence from the HVA α_1 subunits than the divergence between the Ca_V1 and Ca_V2 families (FIG. 2b). Ca_V3 channels are widely expressed in neurons and have important functions in contributing to pacemaking and other repetitive neuronal firing, as well as to subthreshold oscillations[2,16–19].

Association of α_1 subunits with auxiliary subunits. Purification studies showed that L-type[7,20], N-type[21] and P/Q-type[22] calcium channels can all be associated with

auxiliary $\alpha_2\delta$ and β subunits (FIG. 2a). However, in these and other investigations, the association of $\alpha_2\delta$ subunits with VGCC complexes was found to be weaker than that of β subunits, and was dependent on the solubilization conditions that were used to extract the channels[8]. Nevertheless, both the β and $\alpha_2\delta$ subunits markedly enhance the functional expression of cloned HVA channels. Notably, $Ca_V2.3$ (R-type) channels have not been purified yet owing to the lack of a suitable ligand, but most studies show that the $Ca_V2.3$ current amplitude and properties are also influenced by β and $\alpha_2\delta$ subunits[23].

The auxiliary $\alpha_2\delta$ subunits

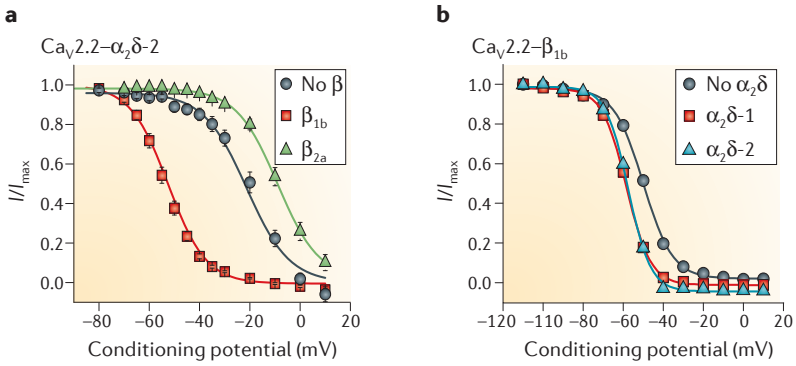
$\alpha_2\delta$ isoforms. Four mammalian $\alpha_2\delta$ subunit genes have been cloned; these are termed *CACNA2D1, CACNA2D2, CACNA2D3* and *CACNA2D4* and encode $\alpha_2\delta$-1, $\alpha_2\delta$-2, $\alpha_2\delta$-3 and $\alpha_2\delta$-4, respectively (for a review, see REF. 24). A number of similar genes have been identified bioinformatically[25], but no studies have been conducted to examine whether the encoded proteins function in the same way as $\alpha_2\delta$ subunits. The $\alpha_2\delta$ subunit genes undergo alternative splicing, which probably expands the $\alpha_2\delta$ subunit functional repertoire, although no particularly divergent properties have been identified to date for the different splice variants[26]. The main $\alpha_2\delta$-1 subunit splice variant that is present in rat brain was found to be different from that in skeletal muscle[27]. Three alternatively spliced regions, known as A, B and C, were then identified from multiple sequence alignments, and five transcripts consisting of different combinations of these alternatively spliced regions were found in mouse brain, skeletal muscle, heart, smooth muscle and aorta[28]. A number of splice variants of the other $\alpha_2\delta$ subunits have been described[29,30].

Processing of $\alpha_2\delta$ subunits. The topology of $\alpha_2\delta$-1 has been determined biochemically, and is thought to be shared with the other three $\alpha_2\delta$ subunits (for reviews, see REFS 24,31). The α_2 subunit was found to be bound to the smaller δ subunit through disulphide bonds. Following the initial cloning of the gene encoding $\alpha_2\delta$-1 from skeletal muscle and N-terminal sequencing of the δ peptide, it was realized that α_2 and δ proteins are expressed from the same gene, which encodes an $\alpha_2\delta$ pre-protein[32]. This pre-protein is post-translationally proteolysed into α_2 and δ proteins[33] by an unknown protease, at an unknown subcellular location. Disulphide bond formation and *N*-glycosylation, at several sites in the protein, occur in the endoplasmic reticulum and Golgi apparatus before proteolytic cleavage[34], and following this cleavage the α_2 and δ moieties then remain associated by inter-subunit disulphide bonding. The residues that are involved in the disulphide bonds that link α_2 and δ moieties in $\alpha_2\delta$-1 have been identified[35].

The $\alpha_2\delta$ subunits have an exofacial N terminus, as indicated by the presence of an N-terminal signal sequence, which directs the protein into the endoplasmic reticulum lumen, where the signal sequence is co-translationally cleaved. These subunits also have a C-terminal hydrophobic domain, which marks them out as type I transmembrane proteins. However, they have very short predicted intracellular sequences beyond this potential transmembrane domain, particularly in the cases of $\alpha_2\delta$-3 and $\alpha_2\delta$-4 (REF. 24). Furthermore, various proteomic programmes predict that some of these $\alpha_2\delta$ proteins are glycosyl-phosphatidylinositol (GPI)-anchored, and this prediction is particularly strong for $\alpha_2\delta$-3 (REF. 34). Indeed, a large amount of biochemical evidence has been obtained that supports the idea that both heterologously expressed and endogenous $\alpha_2\delta$ proteins can form GPI-anchored proteins[34].

Structure of $\alpha_2\delta$ subunits. Single-particle electron microscopic studies of calcium channel complexes that have been purified from skeletal or cardiac muscle have provided low-resolution structures for several subtypes of VGCC. Despite their low resolution, these structures have allowed the tentative identification of

Box 1 | **The definition of a subunit**



A subunit is defined as a protein that co-assembles with others to form a functional complex; for example, a protein that forms part of a multi-subunit enzyme or an ion channel. Such complexes are said to have quaternary structure, which is defined as the arrangement of the subunits in a complex. The assumption, when using the term subunit, is that the protein is a permanent member of the complex. Indeed, an obligate subunit is always present in a complex and is required for function, although several isoforms of the obligate subunit might fulfil the same function. Thus, an α_1 subunit of voltage-gated calcium channels (VGCCs) would be described as an obligate subunit.

The VGCC $\alpha_2\delta$ and β subunits, despite exerting modulatory influences on all high voltage-activated calcium channels, are not involved directly in channel function, and they are usually termed auxiliary or accessory subunits. It remains unclear whether VGCC $\alpha_2\delta$ and β subunits can also be considered as obligate subunits; that is, whether they are always present in all Ca_V1 and Ca_V2 complexes. It is possible that $\alpha_2\delta$ and/or β subunits dissociate from these complexes under certain conditions.

To be a subunit, rather than a chaperone protein that aids folding and trafficking, a protein should exert some distinguishing effects on the function of the complex. This is certainly the case for β subunits, as they exert isoform-specific effects on voltage-dependent properties and channel opening probability (for a review, see REF. 98). For example, the voltage dependence of steady-state inactivation of $Ca_V2.2$–$\alpha_2\delta$-2 is hyperpolarized in the presence of β_{1b} but is depolarized in the presence of β_{2a}, which is palmitoylated (see the figure, part **a**)[140]. The effects of $\alpha_2\delta$ subunits are more subtle, but nevertheless identifiable both in heterologous and homologous expression studies[23,39,59,141,142]. For example, the voltage dependence of steady-state inactivation of $Ca_V2.2$–β_{1b} is hyperpolarized in the presence of $\alpha_2\delta$-1 or $\alpha_2\delta$-2 (see the figure, part **b**)[143].

The distinction between a subunit and an associated protein such as calmodulin, which is involved in downstream signalling, is not always clear. Classically, a subunit would remain bound under all conditions. However, the affinity of calmodulin for its binding sites on the $Ca_V1.2$ carboxyl terminus has been reported to be much greater when it is bound to Ca^{2+} than when it exists as apo-calmodulin[144]. Bars in part **a** show standard error. Part **a** is modified, with permission, from REF. 140 © (2007) Landes Bioscience. Part **b** is modified, with permission, from REF. 143 © (2003) Bentham Science Publishers.

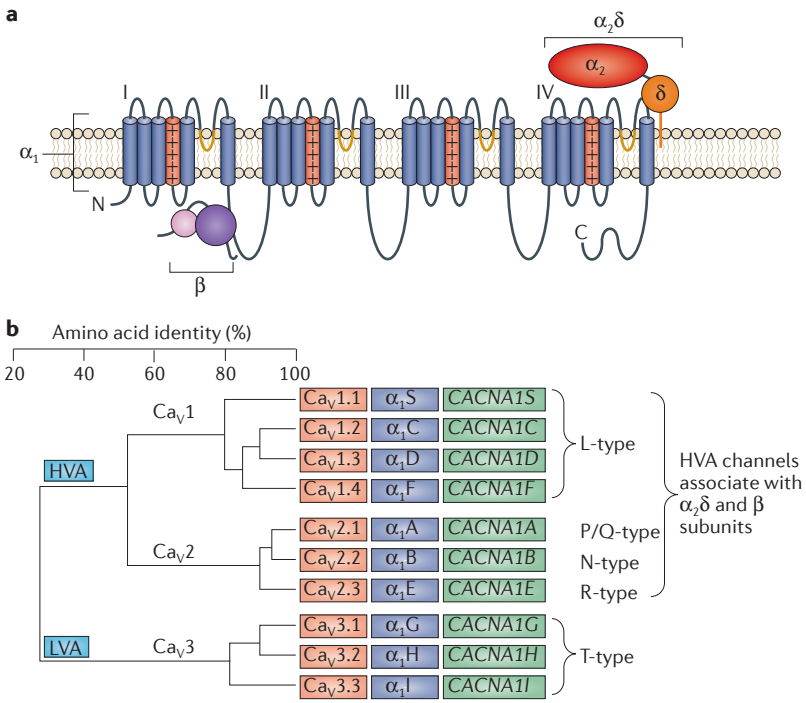


Figure 2 | **Voltage-gated calcium channel subunits. a** | Voltage-gated calcium channel α_1 subunits have 24 transmembrane α-helices, organized into four homologous repeats (I–IV). The fourth transmembrane segment of each repeat (S4; shown in red) has approximately five positively charged amino acids and, together with S1, S2 and S3, this comprises the voltage-sensing domain of the channel. The yellow segments represent the pore loops. β subunits consist of an Src homology (SH3) domain (pink circle) and a guanylate kinase domain (purple circle), connected by a variable linker region. They bind via their guanylate kinase domain to the intracellular linker between domains I and II of the α_1 subunit, with nM affinity. The $\alpha_2\delta$ subunit consists of α_2 (red), which is an extracellular subunit, disulphide-bonded to the δ subunit (orange), which is membrane-associated. The site (or sites) of interaction between the α_1 subunit and the $\alpha_2\delta$ subunit is poorly understood. **b** | α_1 subunits can be divided into three subclasses according to amino acid sequence identity, as shown in the dendrogram (which is based on an alignment of the membrane-spanning regions and pore loops of the α_1 subunits)[17]. The Ca_V1 and Ca_V2 classes are loosely termed high-voltage-activated (HVA) channels, although $Ca_V1.3$ and $Ca_V1.4$ are activated by relatively mid to low voltages. The Ca_V3 α_1 subunits all form low-voltage-activated (LVA) channels. The original names (shown in blue), Ca_V nomenclature (shown in red) and gene names (shown in green) of the α_1 subunits are given. Part **b** is modified, with permission, from REF. 17 © (2003) American Physiological Society.

von Willebrand factor A domain
(VWA domain). These domains are found in many proteins, including integrins, and are generally involved in extracellular protein–protein interactions.

Metal ion-dependent adhesion site motif
(MIDAS motif). A motif located within the von Willebrand factor A domain. It binds a divalent cation, usually Ca^{2+} or Mg^{2+}, to mediate high-affinity interactions with another protein, and is often associated with structural rearrangements.

the positions of the $\alpha_2\delta$ and β subunits within these complexes[36–38]. There is little detailed structural information available, although bioinformatic analysis shows that all $\alpha_2\delta$ subunits contain several recognizable protein domains, including a von Willebrand factor A domain (VWA domain)[25] (FIG. 3a). In general, VWA domains are involved in protein–protein interactions, particularly between the extracellular matrix and cell-adhesion proteins, through a metal ion-dependent adhesion site motif (MIDAS motif)[25], which coordinates a divalent cation, usually Ca^{2+} or Mg^{2+} (REF. 25). Both $\alpha_2\delta$-1 and $\alpha_2\delta$-2 contain a 'perfect' MIDAS motif, in which all five coordinating amino acids are predicted to be present, making it highly likely that a structural rearrangement of the protein complex occurs upon divalent cation-dependent complex formation with a protein ligand[25].

The structure of $\alpha_2\delta$ VWA domains has been modelled by homology with others in the structure database[39] (FIG. 3a). Furthermore, C-terminal to the VWA domain in $\alpha_2\delta$ there are two domains with homology to the extracellular domains of bacterial chemosensing proteins (the so-called bacterial chemosensory-like domains (CSDs)) (FIG. 3b): these have also been termed Cache domains[40]. In bacteria, these proteins are involved in sensing a variety of nutrients, and in plants the ethylene receptor has a similar domain[41].

Distribution of $\alpha_2\delta$ subunits in the nervous system. The $\alpha_2\delta$-1, $\alpha_2\delta$-2 and $\alpha_2\delta$-3 subunits are expressed widely in both the CNS and the peripheral nervous system, with $\alpha_2\delta$-1 being found in many neuronal cell types[42], including dorsal root ganglion (DRG) neurons[43,44]. The $\alpha_2\delta$-1 protein is mainly present in presynaptic terminals and, to a much lower extent, in cell bodies under physiological conditions[44,45]. In rat tissue, the expression of the $\alpha_2\delta$-1 transcript was also found to be loosely correlated with excitatory rather than inhibitory neurons[42]. In contrast to the distribution of $\alpha_2\delta$-1, $\alpha_2\delta$-2 expression is more restricted, and correlates partially with GABAergic neurons, including cerebellar Purkinje neurons[42,46]. The $\alpha_2\delta$-3 protein is expressed throughout rat brain, particularly in the hippocampus, cerebral cortex and caudate putamen[42]. In contrast to the subunits described above, $\alpha_2\delta$-4 is found in specific endocrine tissues and at a low level in the brain[30]. This subunit is also present in neurons in the retina, where it is important for retinal transmission[47,48].

Subcellular distribution of $\alpha_2\delta$ subunits in neurons. The $\alpha_2\delta$ subunits all strongly localize to cholesterol-rich detergent-resistant membrane (DRM) fractions — termed 'lipid rafts' — in transfected cells and in neurons[34,49]. This localization suggests that these subunits may be restricted to specific microdomains in neuronal membranes and may induce an association between their α_1 subunits and such microdomains[49]. In line with this assertion, the mobility of $\alpha_2\delta$-4 (and by inference the retinal L-type channels) in rods and cones has been found to be highly confined to synaptic regions, but to increase transiently on neurotransmitter release, and to show less confinement following lipid raft disruption[50]. The $\alpha_2\delta$ subunits also show rapid and constitutive endocytosis[51,52].

$\alpha_2\delta$-1 is mainly associated with synapses, rather than cell bodies[45], and its presence at the plasma membrane of central primary afferent terminals has been confirmed by electron microscopy[44]. Furthermore, transient overexpression of $\alpha_2\delta$-1, $\alpha_2\delta$-2 and $\alpha_2\delta$-3 subunits in cultured hippocampal neurons led to a large increase in the presynaptic abundance of both $\alpha_2\delta$ and endogenous P/Q-type channels. There was also an increase in the probability of vesicular release in response to a single action potential, an effect that depended on an intact MIDAS motif in $\alpha_2\delta$-1 and $\alpha_2\delta$-2 (REF. 53).

Little immunohistochemical information exists on the subcellular distribution of $\alpha_2\delta$-3 because of a lack of suitable antibodies; however, evidence suggests that the *Drosophila melanogaster* $\alpha_2\delta$-3 subunit homologue,

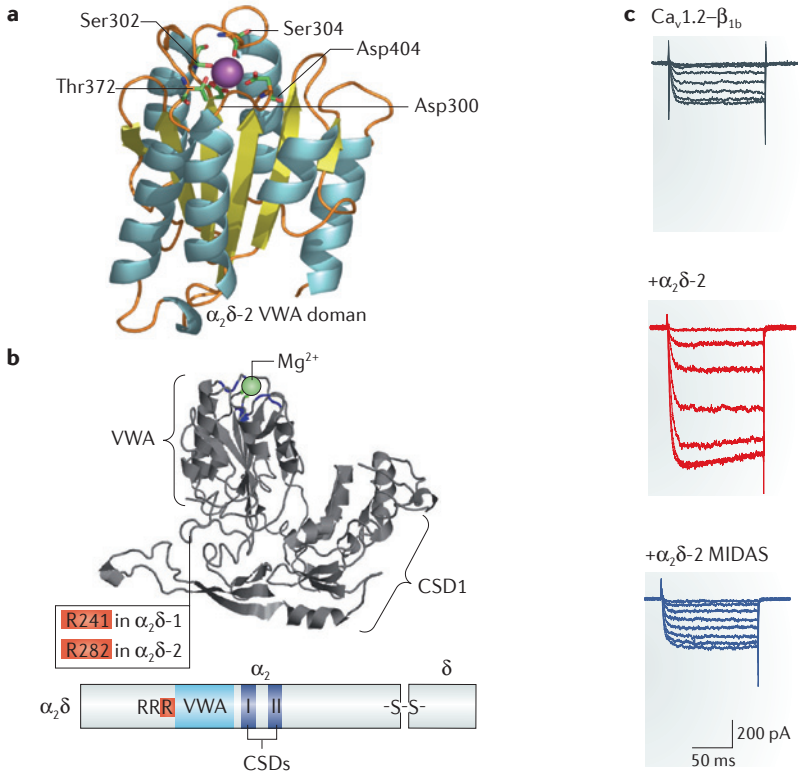


Figure 3 | **Domains in $\alpha_2\delta$ subunits.** **a** | The primary structure of $\alpha_2\delta$ subunits contains a number of domains that can be recognized bioinformatically, one of which is the von Willebrand factor A (VWA) domain. Downstream of this domain lie two bacterial chemosensory-like domains (CSDs; also known as Cache domains)[40]. The structure of the $\alpha_2\delta$-2 VWA domain has been modelled by homology with many other VWA domains for which crystal structures have been determined and placed in the structure database[39]. The predicted α-helices (shown in blue), β-sheet (shown in yellow) and loops (shown in orange) are included. The orientation of the structure is such that the metal ion-dependent adhesion site (MIDAS) motif, consisting of five residues that together coordinate a divalent cation, is at the top. All five MIDAS amino acids — Asp300, Ser302, Ser304, Thr372 and Asp404 — are shown here coordinating Mg^{2+}. Mutation of the first three of these amino acids creates a mutant $\alpha_2\delta$ subunit that has a VWA domain that cannot bind the divalent cation, blocking VWA domain-dependent functions of $\alpha_2\delta$ subunits. To block VWA function in mouse $\alpha_2\delta$-2, the sequence DVSGS was mutated to AVAGA. **b** | Structural model of the VWA domain and the first predicted CSD (CSD1) of rat $\alpha_2\delta$-1. The order of the domains in $\alpha_2\delta$-1 is shown beneath the model. The RRR motif that is key to gabapentin binding is situated on a loop just before the VWA domain. The third Arg in this motif (highlighted in red) has been termed Arg217 (REF. 87), but this numbering is taken from the end of the 24-residue signal sequence and represents Arg241 in the primary rat and mouse $\alpha_2\delta$-1 sequences. The corresponding residue in the mouse $\alpha_2\delta$-2 sequence is Arg282. The sequence of $\alpha_2\delta$-1 encoding the VWA domain and CSD1 (starting at residue Lys232 and finishing at residue Ala650) was submitted to Phyre2 (REF. 170) for structure prediction. The predicted VWA domain structure contains Mg^{2+}, placed using the program 3DLigandSite. The residues predicted to contact the ligand are shown in blue. The CSD1 structure in $\alpha_2\delta$-1 (amino acids 491–607) was predicted with ~99% confidence, and was modelled on six bacterial chemosensory domains (each predicted with >97% confidence), including the extracellular domain of the *Methanosarcina mazei* histidine kinase mmHK1s-z2 and the putative sensory box protein (GGDEF) domain from *Vibrio parahaemolyticus*. **c** | To determine the role of the VWA domain, calcium channel currents were recorded in response to increasing depolarization to between −10 mV and +25 mV in 5 mV steps from a holding potential of −90 mV in transfected tsA-201 cells expressing $Ca_V1.2$–β_{1b} alone (shown by black traces), or in the presence of either wild-type $\alpha_2\delta$-2 (shown by red traces) or $\alpha_2\delta$-2 in which three MIDAS amino acids, Asp300, Ser302 and Ser304, were mutated to Ala residues (shown by blue traces)[39]. Ba^{2+} was used as a charge carrier. The increase in Ba^{2+} current (I_{Ba}) seen in the presence of wild-type $\alpha_2\delta$-2 was not observed when the MIDAS mutant $\alpha_2\delta$-2 was used, implying that the MIDAS site is essential for this function of the $\alpha_2\delta$ subunit. Part **a** and part **c** are reproduced, with permission, from REF. 39 © (2005) National Academy of Sciences.

Straitjacket (STJ), interacts with Cacophony (the α_1 subunit homologue), which is a calcium channel that is involved in active zone localization of calcium channels and presynaptic release[54]. Furthermore, the *Caenorhabditis elegans* $\alpha_2\delta$ subunit (UNC-36) is essential for the correct presynaptic localization of the worm Ca_V2 calcium channel homologue (UNC-2)[55]. These results all indicate that $\alpha_2\delta$ subunits have a role in targeting calcium channels to specific presynaptic sites.

In DRG neurons, $\alpha_2\delta$-1 subunits are also trafficked from the site of synthesis in the cell body, down peripheral as well as central axons[44]. This finding suggests that $\alpha_2\delta$-1 might also affect processes other than calcium channel trafficking; such as axonal regeneration and sprouting at sites of peripheral injury of DRG neurons (FIG. 1b).

Function of $\alpha_2\delta$ subunits in calcium channel complexes. The $\alpha_2\delta$ subunits generally increase the maximum current density for heterologously expressed Ca_V1 and Ca_V2 calcium channels (FIG. 3c) (for a review, see REF. 56). They also affect the biophysical properties of these channels, by increasing their inactivation rate to varying extents. In some studies, expression of particular $\alpha_2\delta$ subunits also hyperpolarized the mid-point potential for steady-state inactivation of the channels[34,39,51] (BOX 1).

The main mechanisms underlying the $\alpha_2\delta$ subunit-induced increase in maximum current density are probably an increase in the plasma membrane expression of Ca_V1 and Ca_V2 complexes coupled with a decrease in their turnover[39,57]. Although it is still unclear how the $\alpha_2\delta$ subunits confer their effects, their MIDAS motif is essential for these mechanisms[39] (FIG. 3c). Mutation of this motif markedly reduces the functionality of both $\alpha_2\delta$-1 (REF. 53) and $\alpha_2\delta$-2 (REF. 39) subunits in terms of their ability to increase calcium currents in expression systems. Moreover, the MIDAS mutant of $\alpha_2\delta$-2 causes intracellular retention of α_1 subunits[39].

The point at which $\alpha_2\delta$ subunits exert their influence on the trafficking of the calcium channel complex remains to be determined, but it is likely that they interact with one or more exofacial loops of the α_1 subunit. For example, it has been shown that the α_2 subunit of $\alpha_2\delta$-1 binds to domain III of $Ca_V1.1$, as one site of interaction[58]. Previous evidence has also shown that the transmembrane segment of the δ subunit interacts with α_1 subunits[59]. However, this evidence may need to be reconsidered in light of the finding that $\alpha_2\delta$ subunits can form GPI-anchored proteins[34]. The trafficking of $\alpha_2\delta$ subunits themselves has also been studied[34,39,49,51,52], and much evidence indicates that for this process to occur, $\alpha_2\delta$ subunits must interact with other cellular trafficking proteins, which remain to be identified.

Non-calcium channel roles of $\alpha_2\delta$ subunits. The $\alpha_2\delta$ subunits are only loosely associated with calcium channel complexes, and a proportion of free $\alpha_2\delta$ subunits can be isolated from neural tissue by column chromatography[8,60]. This finding supports the possibility that these proteins fulfil other functions. One indication that $\alpha_2\delta$ subunits may serve other purposes comes from the

Synaptogenesis
Synaptogenesis involves the formation of synapses between a presynaptic terminal and a postsynaptic element. It results in the close apposition of presynaptic active zones, which contain calcium channels and vesicular release sites, with postsynaptic membranes, which contain neurotransmitter receptor ion channels and other postsynaptic proteins.

finding that the genes encoding $\alpha_2\delta$-2 (REF. 61) and $\alpha_2\delta$-3 (REF. 62) may be associated with tumour susceptibility, as they show reduced expression in some cancer cells[61]. Furthermore, overexpression of $\alpha_2\delta$-2 caused apoptosis in small-cell lung cancer cell lines[63]. In addition, $\alpha_2\delta$-1 subunits may be involved in the development and migration of myotubes independently of their role in the muscle calcium channel complex (BOX 2).

In neurons, $\alpha_2\delta$-3 subunits have been found to have a role in synaptogenesis in *D. melanagaster* embryos, independent of their association with calcium channels[64]. In this study, the synaptic boutons of motor neuron terminals failed to develop normally in *stj* ($\alpha_2\delta$-3)-knockout flies, although presynaptic specializations were present, and spontaneous miniature excitatory postsynaptic potentials could be recorded. However, no evoked synaptic transmission was observed, implying an absence of Cacophony from the active zone. In flies with normal STJ expression but no Cacophony expression, boutons developed normally, indicating that bouton morphology requires a process involving STJ that is independent of its calcium channel-related function[64]. This finding suggests that $\alpha_2\delta$-3 proteins are multifunctional, being involved both in correct calcium channel localization at the synapse and in other processes associated with synaptogenesis (FIG. 4a). Related to this, $\alpha_2\delta$-1 has been found to be involved in mammalian excitatory synaptogenesis through binding to extracellular matrix proteins of the thrombospondin family[65]. Thrombospondin- and astrocyte-induced excitatory synapse formation was found to require postsynaptic $\alpha_2\delta$-1 (REF. 65) (FIG. 4b), which is in interesting contrast to the predominantly presynaptic localization of $\alpha_2\delta$-1 in adult neurons[45]. It will be of interest to determine whether the $\alpha_2\delta$ subunit isoforms have interchangeable functions or are involved in the formation of different subtypes of synapse. Indeed, thrombospondins also bind to a number of other proteins, and this might implicate $\alpha_2\delta$ proteins in many processes, both in the nervous system and elsewhere[66].

$\alpha_2\delta$ subunits and disease

The $\alpha_2\delta$ subunits have roles in various disorders. For example, mutations in *CACNA2D1* are associated with several forms of cardiac dysfunction, including Brugada[67] and short QT[68] syndromes. Moreover, a spontaneous mouse mutation and human mutations in *CACNA2D4* (which encodes the $\alpha_2\delta$-4 subunit) have been identified that show similar phenotypes of autosomal recessive cone dystrophy and night blindness[47,48]. Most recently, a splice site mutation in *CACNA2D3* was found to be a probable susceptibility gene for autism spectrum disorders[69].

$\alpha_2\delta$-1 subunits have also recently been identified to interact with the prion protein (PrP)[70]. Interaction with misfolded mutant PrP molecules that accumulate in the endoplasmic reticulum resulted in intracellular retention of $\alpha_2\delta$-1. In transgenic mice expressing a pathogenic variant of PrP, there was a reduction of functional calcium currents in cerebellar granule neurons, accompanied by impaired glutamatergic neurotransmission in the cerebellum. These results provide further evidence that $\alpha_2\delta$ can interact with other proteins as well as $Ca_V\alpha_1$ subunits, and this may influence calcium channel function. It will be of interest in the future to examine the extent of the role of native PrP in calcium channel function and trafficking.

Perhaps the two best-studied links between $\alpha_2\delta$ subunits and disease concern neuropathic pain and epilepsy, which are discussed below.

Neuropathic pain. Experimental peripheral nerve injury results in an increase in the level of $\alpha_2\delta$-1 mRNA in damaged sensory neurons (DRGs), as has been shown by *in situ* hybridization[43], microarray analysis[71] and quantitative PCR[44]. There is a corresponding increase in $\alpha_2\delta$-1 levels in DRG cell bodies and at their presynaptic terminals in the spinal cord, as determined by western blot analysis[72] and immunohistochemistry[44]. By contrast, $Ca_V2.2$ mRNA and protein levels are not consistently reported to be upregulated following sensory nerve damage[71,73]. This finding suggests that upregulated levels of $\alpha_2\delta$-1 enhance $Ca_V2.2$ trafficking and presynaptic function, although they may also have other functions, other than in calcium channels, as described above. Furthermore, $\alpha_2\delta$-1-overexpressing mice show a neuropathic phenotype of hyperalgesia and tactile allodynia in the absence of nerve injury[73], indicating that $\alpha_2\delta$-1 is instrumental to the excitability of DRG neurons and the expression of neuropathy.

A study recently identified *stj* and *Cacna2d3* as 'pain genes' (REF. 74), because mutant *D. melanogaster* and mice lacking these respective genes showed impairment in the avoidance of noxious heat. This phenotype resulted from impaired central processing rather than any effects at the level of sensory input. Interestingly, two

Box 2 | Role of $\alpha_2\delta$-1 subunits in skeletal and cardiac muscle

Skeletal-muscle transverse (T) tubules are invaginations of the plasma membrane into the muscle that contain a highly ordered array of calcium channels and allow a depolarizing signal to penetrate rapidly throughout the muscle. At this location, the $\alpha_2\delta$-1 protein is found in association with the L-type calcium channel complex ($Ca_V1.1$, β_{1a} and γ_1)[145]. The $\alpha_2\delta$-1 isoform is also strongly expressed in cardiac and smooth muscle, and is probably the main $\alpha_2\delta$ subunit that is associated with $Ca_V1.2$ in these tissues[146,147].

The role of $\alpha_2\delta$-1 in skeletal muscle remains unclear. In the T-tubule junction with the sarcoplasmic reticulum, the $Ca_V1.1$ channel complexes form tetradic structures, which are visible using electron microscopy and are juxtaposed to ryanodine receptors on the sarcoplasmic reticulum[148,149]. Surprisingly, although partial loss of $\alpha_2\delta$-1 expression through application of small interfering RNAs (siRNAs) caused a marked increase in the rate of activation of the L-type calcium current in myotubes, it had little effect on excitation–contraction coupling[142]. More extensive knockdown of this subunit, by viral infection of siRNA in myotubes, led to a similar effect on current kinetics, but the size and spacing of the tetradic Ca_V particles was unaffected by loss of $\alpha_2\delta$, indicating that the visible particles probably represent the α_1S ($Ca_V1.1$) subunit itself[141].

Three different studies have found contrasting effects of loss of $\alpha_2\delta$-1 on skeletal-muscle development. In one study, $\alpha_2\delta$-1 was found not to be necessary for myotube growth or for the differentiation of myoblasts to form myotubes[141]. However, a study involving developing myocytes showed that when the expression of $\alpha_2\delta$-1 was reduced following siRNA treatment, the migration, attachment and spreading of myoblasts was impaired, although the L-type calcium current remained unaffected[150]. Nevertheless, in $\alpha_2\delta$-1 knockout mice, which have a cardiac phenotype comprising reduced cardiac calcium currents and decreased myocardial contractility, the skeletal-muscle structure and function seems to be grossly normal[151].

Gabapentinoid
The drugs gabapentin and pregabalin are collectively termed gabapentinoids, which are also known as $\alpha_2\delta$ ligand drugs.

intronic single nucleotide polymorphisms (SNPs) within the *CACNA2D3* gene were associated with reduced acute and chronic pain in humans, although the mechanism behind this difference is unknown[74].

Epilepsy. The first evidence that the $\alpha_2\delta$ subunits might be involved in epilepsy was provided when these proteins were discovered to be the site of action of gabapentin (see below), a compound that was already in use as an add-on drug to improve the control of epileptic seizures. Subsequently, three strains of spontaneous mouse mutants — *ducky, ducky^{2J}* and *entla* — were found to harbour mutations in *Cacna2d2*. These mice exhibit cerebellar ataxia as well as absence and/or generalized epilepsy, as do mice with a targeted *Cacna2d2* gene deletion[46,75–77]. Despite these findings in mice, to date no human mutations in the genes encoding $\alpha_2\delta$ subunits have been reported to be associated with epileptic phenotypes. In a study looking for SNPs in ion channel genes that might be associated with idiopathic epilepsy, the coding regions of multiple genes were screened in more than 100 patients with this condition and matched controls[78]. This study revealed that there were multiple SNPs in many ion channel genes, with *CACNA2D1* and *CACNA2D2* being two of the genes showing a large number of SNPs. However, perhaps surprisingly, the number and combination of SNPs was similar in both patient and control groups, so the presence of SNPs in the ion channel genes studied cannot account for the disease phenotype of these patients, although the authors suggest that in the future, exome sequencing might be able to predict the effectiveness of specific anti-epileptic drugs for individual patients.

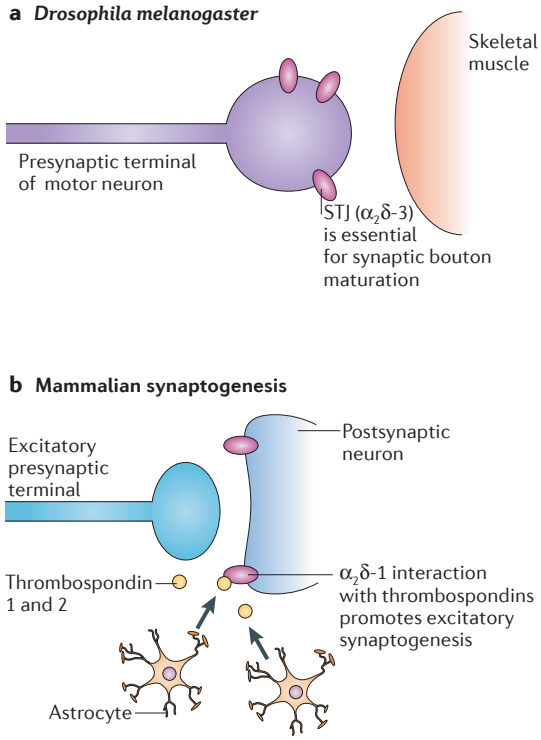


Figure 4 | **New roles for $\alpha_2\delta$ subunits may affect neuronal function independently from their role as calcium channel subunits. a** | The *Drosophila melanogaster* homologue of $\alpha_2\delta$-3 (Straitjacket (STJ)) is involved in synaptic bouton formation[64]. In the absence of presynaptic STJ, synaptic contacts with the skeletal muscle are still present, but a mature bouton is not formed. This function could be replicated with the α_2 moiety of STJ alone[64]. **b** | The interaction of $\alpha_2\delta$-1 and thrombospondins promotes synaptogenesis of excitatory synapses[65]. Thrombospondins are extracellular matrix proteins that have synaptogenic activity and are secreted from various cell types, including astrocytes. Overexpression of $\alpha_2\delta$-1 in postsynaptic cells was found to cause an increase in synapse formation[65]. Both of these new functions suggest that $\alpha_2\delta$ subunits are likely to be involved in interactions with proteins other than calcium channels on the cell surface or in the extracellular matrix.

$\alpha_2\delta$ subunits as therapeutic targets

Targets of gabapentinoid drugs. Gabapentin (2-(1-(aminomethyl)-cyclohexyl)acetic acid) was synthesized as a rigid analogue of the inhibitory neurotransmitter GABA, with the aim of developing therapeutic agents of use in the treatment of epilepsy. Gabapentin was indeed found to be an efficacious antiepileptic drug, particularly in combination with other first-line drugs[79]. Pregabalin (3-(aminomethyl)-5-methyl-hexanoic acid) was synthesized as part of a series of drugs that were designed to affect GABA metabolism, but despite being identified as a potent anti-seizure drug in animal models, its efficacy was found not to be related to its action on GABA synthesizing or metabolizing enzymes[80]. Thus, the mechanism of action of both of these drugs was initially unclear, as the consensus was that they did not have any notable effect on GABA receptors, metabolism or transport (for reviews, see REFS 80,81).

Both drugs were subsequently found to be effective treatments for various forms of neuropathic pain, including diabetic and chemotherapy-induced neuropathy and post-herpetic and trigeminal neuralgia, although they had a relatively slow onset of action[82–84]. The effect of these drugs was also found to be state-dependent, in that they have little effect on acute pain perception in naive animals or humans, but they are effective in chronic neuropathic pain[85], albeit in a subset of patients[83,84].

A purification study in pig brain led to the surprising discovery that the ^{3}H-gabapentin binding site corresponded to $\alpha_2\delta$-1 (REF. 60). A subsequent study showed that ^{3}H-gabapentin could also bind $\alpha_2\delta$-2 (REF. 86). Several amino acids have been shown to be involved in the binding of gabapentinoid drugs to $\alpha_2\delta$ subunits; one of these being the third arginine (R) in an RRR motif, situated just before the VWA domain[49,87]. In a homology model of the tertiary structure of specific domains identified within $\alpha_2\delta$-1, the loop containing the RRR motif is juxtaposed to CSD1 (FIG. 3b). It is attractive to suggest that the basis of the ability of $\alpha_2\delta$ subunits to bind gabapentinoid drugs relates to the presence of these ancestral chemosensory ligand-binding domains (FIG. 3b), and that an endogenous modulator might also bind to these subunits. Some evidence indeed exists that one or more endogenous substances can bind $\alpha_2\delta$ subunits and directly or

allosterically inhibit gabapentin binding[88]. The binding affinity for ^{3}H-gabapentin increases as the $\alpha_2\delta$ protein is purified or dialysed[49,89], possibly because of loss of putative endogenous modulators. Interestingly, large neutral amino acids, such as leucine, were found to bind to the purified ^{3}H-gabapentin receptor (now known to correspond to both $\alpha_2\delta$-1 and $\alpha_2\delta$-2) from brain[89], and these amino acids might represent endogenous modulators. It is also tempting to speculate that the association between gabapentin (or an endogenous ligand) and its $\alpha_2\delta$ binding site, which includes the RRR motif, might influence the function of the VWA domain.

Mechanism of action of the gabapentinoid drugs. Despite binding to $\alpha_2\delta$ subunits, gabapentin has been found to have either no acute effect or only a small acute inhibitory effect on expressed calcium currents[51], those in brain[49,90,91] and those in DRG neurons[51,92,93]. Nevertheless, it has recently been shown that the binding of gabapentin and pregabalin to $\alpha_2\delta$-1 subunits is essential for their therapeutic effect in experimental models of neuropathic pain[87]. Furthermore, it has also been reported that, although there was no effect of gabapentin on calcium channel currents in wild-type mouse DRG neurons, the currents in DRG neurons from $\alpha_2\delta$-1-overexpressing mice were sensitive to inhibition by gabapentin[73], suggesting that upregulation of $\alpha_2\delta$-1 in neuropathic pain may render these drugs effective.

Gabapentin has also been reported to disrupt the interaction between $\alpha_2\delta$-1 and thrombospondins *in vitro* and, as a result, interfere with synaptogenesis, although it does not affect pre-formed synapses[65]. This finding could have implications for the prolonged use of this class of drug, if it is confirmed. However, several studies have shown that there is no significant excess of birth defects in babies following chronic gabapentin exposure *in utero*[94,95], indicating that synaptogenesis is unlikely to be affected *in vivo* by concentrations of this drug used in clinical practice.

Recent studies have revealed that chronic application of gabapentin decreases the cell-surface localization of $\alpha_2\delta$ and α_1 subunits, and correspondingly reduces calcium channel currents, both in cell lines and in DRG neurons[51]. Gabapentin also inhibits post-Golgi trafficking of $\alpha_2\delta$-2 in a manner that is occluded by dominant-negative RAB11, which disrupts trafficking through the recycling endosome compartment[52]. In a complementary *in vivo* study, chronic application of pregabalin, at the same time as alleviating neuropathic hyperalgesic responses in spinal-nerve ligated rats, markedly reduced the increase of $\alpha_2\delta$-1 in the presynaptic terminals of the injured DRGs in the dorsal horn *in vivo,* an effect that may result from impaired trafficking[44].

Auxiliary β subunits

Structure of β subunits. The gene for the skeletal muscle β subunit isoform, subsequently termed β_{1a}, was the first VGCC β subunit gene to be cloned[96]. Three further β subunit genes (encoding β_2, β_3 and β_4) were then cloned and a neuronal splice variant of β_1 was also identified, termed β_{1b} (for a review, see REF. 56). The β subunits are cytoplasmic proteins that bind to the proximal part of the intracellular loop between domains I and II of the Ca_V1 and Ca_V2 α_1 subunits. This 18-amino-acid binding motif is termed the α-interaction domain (AID)[97] (FIG. 2a). Several well-conserved amino acids within the AID were found to be crucial for binding to β subunits, including a key tryptophan and tyrosine[97]. There is also evidence that β subunits interact with other regions on calcium channel α_1 subunits. A comprehensive review on β subunit structure and function has recently been published[98], so only recent developments in this field are discussed here.

A modelling study initially revealed that all β subunits contain a conserved Src homology 3 (SH3) domain and a guanylate kinase-like domain[99]. These conserved domains are flanked by regions of variable sequence and length, both between the β isoforms and between splice variants of each isoform. The presence of SH3 and guanylate kinase domains placed β subunits in the membrane-associated guanylate kinase (MAGUK) protein family, which also includes postsynaptic density protein 95 (PSD95)[100], a protein that is involved in ion channel clustering, among other functions.

The isolated SH3 and guanylate kinase domains of β subunits were found to interact with each other as a functional unit[101]. Three structural studies solved the crystal structure of these conserved domains in different β subunits[102–104]. These studies all showed that the AID peptide bound within a deep groove on the guanylate kinase domain[103], and that the residues that were previously shown to be important for binding to β subunits[97] were found to make several interactions within this binding groove (FIG. 5a). In the intact I–II linker, the α-helical structure of the AID, which is imposed by binding to the β subunit, is predicted to continue to the end of the sixth segment (S6) in transmembrane domain I (REF. 102). Thus, β subunits may act as chaperones to induce correct folding of the I–II linker. In addition, when the β subunit is associated with the AID region, it may directly interact with other parts of the channel that are involved in gating, such as the base of transmembrane segments that form the pore[103].

Several recent pieces of evidence now exist that suggest that β subunits can form dimers. β subunits are able to interact in a yeast two-hybrid screen, which may indicate that the SH3 domain from one β subunit can associate with the guanylate kinase domain of another subunit, at least *in vitro*[105]. β subunit oligomerization has also been shown to occur in intact vascular smooth muscle cells[106]. Furthermore, homodimerization of β subunit SH3 domains has been demonstrated to result in endocytosis of $Ca_V1.2$ (REF. 107). It will be of interest to examine whether this has relevance to the function of β subunits in neurons.

β subunits and VGCC function. β subunits enhance the functional expression and exert a major influence on the biophysical properties of the Ca_V1 and Ca_V2 channels (FIG. 5b). Two processes have been proposed to account for the increase in current that is observed in VGCCs, including these subunits. β subunits generally

hyperpolarize the voltage-dependence of activation, and also increase the maximum open probability of the channel, which will increase current through individual channels and therefore result in increased macroscopic current density[108–110]. However, β subunits have also been found to increase the number of channels that are inserted into the plasma membrane, as determined by gating charge measurements, imaging and biochemical experiments[111–117]. However, increased membrane insertion of channels in the presence of a β subunit has not been observed in all studies[118].

It was originally postulated that β subunits enhanced the trafficking of calcium channels by masking an endoplasmic reticulum retention signal on the I–II linker[114,119], although no specific motif could be identified[119]. Mutation of Trp to Ala in the I–II linker AID motif of $Ca_V2.2$ prevents the functional interaction between α_1 and the β subunit[115], and this mutant can therefore be used to probe β subunit function (FIG. 5b,c). Interestingly, this $Ca_V2.2$ Trp391Ala mutant has a shorter lifetime than wild-type $Ca_V2.2$ because of a more rapid rate of degradation, which is blocked by proteasomal inhibitors[120] (FIG. 5c). It was proposed that lack of interaction with a β subunit results in increased polyubiquitylation and consequent proteasomal degradation, rather than specific retention in the endoplasmic reticulum[114], for both N-type[120] and L-type calcium channels[121]. Thus, induced α-helix formation in the proximal I–II linker and protection from proteasomal degradation may represent two aspects of a general mechanism of action of β subunits to protect the α_1 subunit from interaction with an endoplasmic reticulum-associated E3 ubiquitin ligase and subsequent proteasomal degradation, and hence allow forward trafficking of the channels (FIG. 6).

N-, P/Q- and R-type calcium currents can be inhibited by activation of G protein-coupled receptors (GPCRs) that are linked to $G_{i/o}$. This inhibition is mediated by Gβγ and is classically observed to be voltage-dependent (for a review, see REF. 56); that is, inhibition can be relieved by depolarization, which is thought to result from Gβγ unbinding. Although Gβγ binds to a site on the I–II linker that overlaps with the binding site of β subunits, and was initially thought to compete with the β subunit for binding[122], the absence of the β subunit does not prevent G-protein modulation of calcium

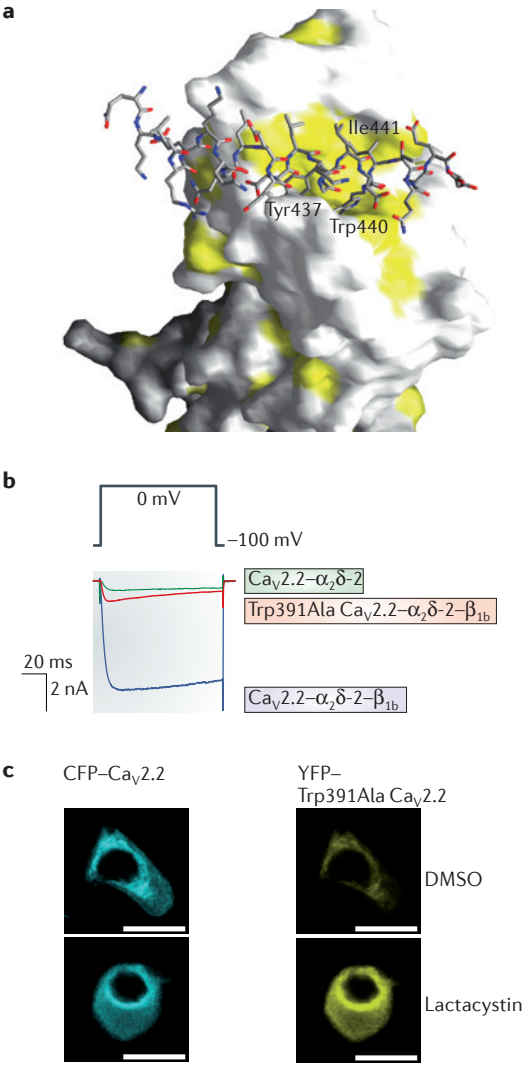


Figure 5 | **β subunit interactions and effects on calcium channel.** β subunits all have two highly conserved structural domains: an Src homology domain 3 (SH3) and a guanylate kinase domain. **a** | Structure of the binding groove in the guanylate kinase domain of β_3 subunits, into which the I–II linker α-interaction domain (AID) peptide from $Ca_V1.2$ is docked[171]. A tryptophan in the I–II linker peptide (Trp440 in $Ca_V1.2$) is essential for β subunit binding. **b** | Mutation of the corresponding tryptophan to alanine (Trp391Ala) in $Ca_V2.2$ was performed to examine the effect of disrupting the normal binding of β subunits to this channel. Compared with tsA-201 cells expressing $Ca_V2.2$–$\alpha_2\delta$-2–β_{1b} (shown by the blue trace), cells expressing Trp391Ala $Ca_V2.2$–$\alpha_2\delta$-2–β_{1b} (shown by a red trace) showed a marked reduction in $Ca_V2.2$-mediated calcium currents that were evoked by a step from –100 mV to 0 mV. Moreover, cells expressing Trp391Ala $Ca_V2.2$–$\alpha_2\delta$-2–β_{1b} showed only slightly greater currents than those expressing $Ca_V2.2$–$\alpha_2\delta$-2 with no β subunit (shown by a green trace)[115]. **c** | The mechanism behind the effect of β subunits on calcium channel currents was probed using fluorescent protein-tagged $Ca_V2.2$ channels to examine their level of expression. Yellow fluorescent protein (YFP)–Trp391Ala $Ca_V2.2$ levels are lower than cyan fluorescent protein (CFP) $Ca_V2.2$ levels when they are expressed together in cultured sympathetic neurons (top panels). This differential expression results from the inability of the mutant channel to associate with β subunits and, as a result, it more readily undergoes proteasomal degradation. This conclusion was reached because the differential expression is reversed by proteasome inhibitors, including lactacystin (bottom panels), compared with dimethylsulfoxide (DMSO) vehicle control (upper panels)[120]. Scale bars represent 20 μm. Part **a** is reproduced, with permission, from REF. 171 © (2004) Elsevier. Part **b** is modified, with permission, from REF. 115 © (2005) Society for Neuroscience. Part **c** is reproduced, with permission, from REF. 120 © (2011) The American Society for Biochemistry and Molecular Biology.

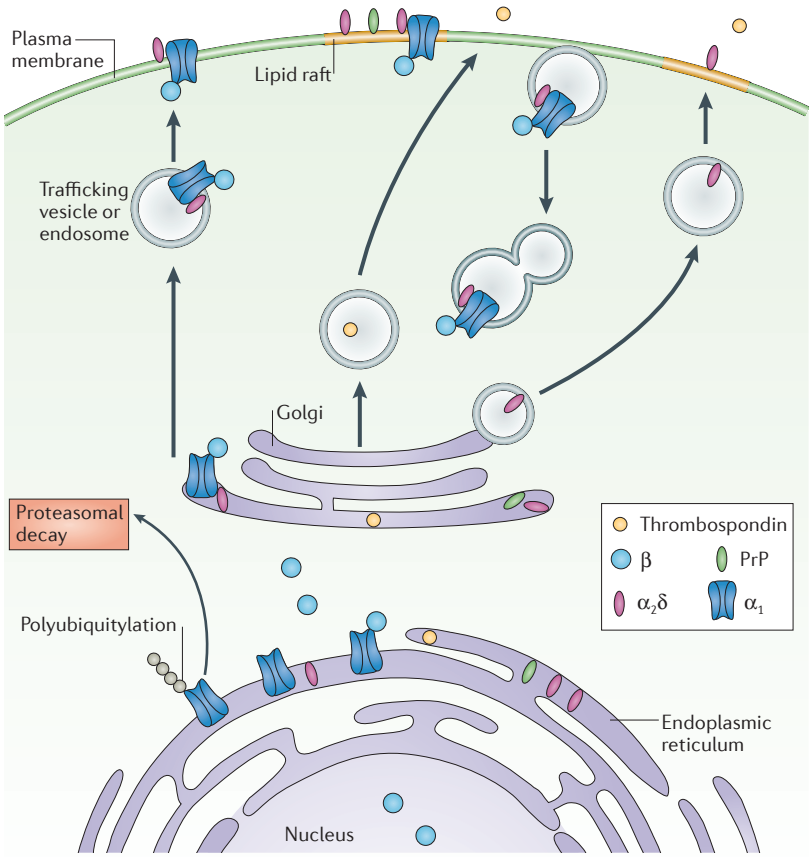


Figure 6 | **Overview of the effects of $\alpha_2\delta$ and β subunits on calcium channel trafficking.** It is proposed that cytoplasmic voltage-gated calcium channel (VGCC) β subunits interact with the nascent VGCC α_1 subunits that are synthesized on the endoplasmic reticulum, and protect them from polyubiquitylation and subsequent proteasomal degradation. β subunits also promote folding of the α_1 subunit I–II linker, and by both these means they promote forward trafficking of VGCCs out of the endoplasmic reticulum and towards the plasma membrane. The $\alpha_2\delta$ subunit may associate with α_1 or β subunits at a later stage; for example, in the Golgi apparatus, to further promote forward trafficking or reduce endocytosis of the calcium channel complex. $\alpha_2\delta$ subunits can also reach the plasma membrane in the absence of the calcium channels and are present in 'lipid raft' fractions that are associated with the plasma membrane[49]. A pool of $\alpha_2\delta$ subunits is therefore likely to exist separately from calcium channel complexes, and may interact with molecules such as prion protein (PrP) in the endoplasmic reticulum or elsewhere[70]. $\alpha_2\delta$-1 subunits may also interact with the extracellular matrix proteins thrombospondins[65]. β subunits are also likely to exist in excess of the number of functional VGCCs, and may have separate, non-VGCC roles; for example, in the nucleus.

channels, although it does abolish the voltage dependence of the process; that is, the G-protein modulation of $Ca_V2.2$ cannot be relieved by depolarizing voltage steps[109]. Furthermore, the Trp391Ala $Ca_V2.2$ mutant channel, which does not bind to β subunits, does not exhibit voltage-dependent G-protein modulation, thus confirming the key role of β subunits in this process[115]. Furthermore, if β subunit is dialysed out of cells, the subsequent re-addition of β subunit protein reinstates voltage-dependent G-protein modulation[123]. Thus, one key function of β subunits is to limit Gβγ-mediated inhibition at depolarized potentials[56].

VGCC β subunits are also important targets for second messengers through phosphorylation by protein kinase A[124], Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)[125] and the phosphoinositide 3-kinase (PI3K)–AKT pathway[126]. β_{2a} subunit phosphorylation by the PI3K–AKT pathway enhanced membrane expression of $Ca_V2.2$ and $Ca_V1.2$ channels[126]. The mechanism underlying this effect was subsequently found to involve a reduction in $Ca_V1.2$ degradation[127]. The C terminus of β_{2a} also directly binds to CaMKII (REF. 125), and this binding mediates, through phosphorylation of a specific residue in the C terminus of β_{2a}, the facilitation of $Ca_V1.2$ currents by CaMKII in heart. Thus, β subunits may act to coordinate multiple phosphorylation-dependent actions on calcium channels. Furthermore, β_{2a} that is membrane-associated by palmitoylation shows enhanced functional interaction with calcium channel α_1 subunits[115], presumably because of its increased effective concentration at the plasma membrane. This lipid modification of β_{2a} also reduces the phosphatidylinositol bisphosphate-mediated inhibitory modulation of calcium channels[128].

Several members of the REM and GEM/KIR (RGK) family of small GTP-binding proteins have been shown to inhibit HVA calcium channels, although the underlying molecular mechanism and physiological relevance of this inhibition are still unclear[129,130]. RGK proteins can associate directly with VGCC β subunits (for a review, see REF. 98), but whether this binding is required for their inhibitory action is debated. It has been suggested recently that GEM binds directly to, and inhibits, α_1 subunits on the plasma membrane that are associated with a β subunit[131]. Moreover, although calcium currents in cultured hippocampal neurons were inhibited by overexpression of REM2, endogenous REM2 was found to be at very low levels, and its knockdown had no marked effect on calcium currents[132], suggesting that there is no tonic inhibitory effect of native REM2 in these cells.

Although binding of the AID region of Ca_V1 and Ca_V2 with the guanylate kinase domain of β subunits represents a high-affinity interaction, other domains in β subunits also influence calcium channel function. These findings suggest that, once tethered on the AID motif, the β subunits interact at several sites on the α_1 subunit[133]. Furthermore, β subunits, in addition to interacting with α_1 subunits, may also have a role in anchoring them at presynaptic sites by forming a bridge with other proteins. The presynaptic active zone protein RIM1 (RAB3-interacting molecule 1) binds, through its C terminus, to the SH3–guanylate kinase core domain of β subunits[134]. By this means, RIM1 suppresses inactivation of both the Ca_V1 and Ca_V2 channels, and thus enhances neurotransmitter release, both by augmenting flux through presynaptic calcium channels and by anchoring the channels close to release sites[134,135]. RIM1 also interacts with RIM-binding protein, which was found to bind directly to α_1 subunits[136]. RIM-binding protein is essential for vesicular release of neurotransmitters, and surrounds a central core of calcium channels in *D. melanogaster* neuromuscular junction active zones[137]. Thus, β subunits may participate in targeting calcium channels to active zones.

Box 3 | **Phenotypes of β subunit knockout animals reveal new functions**

Results from β subunit knockout animals have revealed new functional roles for β subunits. Several isoforms of β subunits are present in many cell types, allowing for compensation in terms of calcium channel function[98,152]. However, β_1 is the only isoform in skeletal muscle. Excitation–contraction coupling is absent from β_1 null mice, and these mice die at birth[153]. It has been shown recently that the loss of functional skeletal muscle dihydropyridine receptors (DHPRs) that is induced by knockout of β_1 leads to defects in the pre-patterning of nicotinic acetylcholine receptors on the muscle, as well as increased neuromuscular innervation[154].

The function of the β_1 subunit has also been investigated in a zebrafish β_1 knockout (*relaxed*), in which the function of the skeletal muscle is disrupted. This phenotype can only be rescued by expression of β_{1a}, as other β subunits — while supporting some restoration of $Ca_V1.1$ charge movement, indicative of the channel complex being in the plasma membrane — do not support the formation of $Ca_V1.1$ channel complexes in the typical tetrads, which result from juxtaposition of the calcium channels with ryanodine receptors in a tetrameric array[155]. By contrast, the rescue of tetrad formation by β_{1a} was associated with the restoration of intracellular calcium release, and the re-establishment of motility in the *relaxed* zebrafish larvae. This finding suggests that β_{1a} has unique motifs that allow the channel complex to interact with the ryanodine receptors, or that the other β subunits, perhaps because they have longer C termini, inhibit the formation of tetrads.

Global deletion of β_2 is also embryonic lethal, resulting from cardiovascular dysfunction[156]. By contrast, mice in which a knockout of β_2 is restricted to the CNS show impaired vision and altered retinal morphology[157]. They have also recently been shown to be deaf, revealing an essential role for this β subunit in regulating $Ca_V1.3$ function in inner hair cells[158].

Furthermore, *lethargic* mice, in which the β_4-encoding gene (*Cacnb4*) is disrupted, show cerebellar ataxia and absence seizures[159], and β_3-null mice show altered pain perception, particularly to inflammatory stimuli[160]. Both β_3- and β_4-null mice also have impaired receptor-mediated intracellular Ca^{2+} responses in T lymphocytes, and a reduction in translocation into the nucleus of the protein nuclear factor of activated T cells (NFAT)[161]. Expression of $Ca_V1.4$ protein was observed in T lymphocytes, and this was reduced in β_3-null mice[162], agreeing with a proposed role for β subunits in increasing calcium channel stability[120,121]. Furthermore, in contrast to previous findings[161], small calcium currents have recently been observed in mouse T lymphocytes that are lost in lymphocytes from $Ca_V1.4$ knockout mice[163]. This reveals a new role for calcium channels in these non-excitable cells.

Surprisingly, β_3 knockout mice showed elevated NMDA receptor (NMDAR)-mediated currents and NMDAR-dependent long-term potentiation[164]. The results suggest that β_3 subunits normally negatively regulate NMDAR activity, although whether this involves a direct interaction has not yet been determined[164]. Furthermore, an essential role for β_4 subunits has been identified in early embryonic development of zebrafish[165], which was suggested to be independent of calcium channels. It will be of interest to determine the pathways involved in this new β_4 function.

Effects of β subunits on gene transcription. Gene knockout studies have revealed both novel and VGCC-associated roles for β subunits (BOX 3). Furthermore, several studies have found that β subunits have effects in the nucleus, although some reports of nuclear localization using green fluorescent protein (GFP)-tagged constructs may result from the tendency of GFP to dimerize, combined with the size restrictions represented by the nuclear pores, trapping proteins in the nucleus.

A short splice variant of β_4 (β_{4c}) was expressed together with other β_4 splice variants in brain, but was found to be the only β_4 splice variant present in the chick cochlea[138]. β_{4c} was found in the nucleus, and was found to bind to the nuclear protein chromobox protein 2 (also known as heterochromatin protein 1γ)[138]. β_{4c} was found to reduce the gene silencing that is caused by this chromatin-binding protein, and in this way it was able to regulate transcription. β_{4c} has little effect on calcium currents, which is to be expected as it lacks most of the guanylate kinase domain. In a related result, a yeast two-hybrid approach revealed that β_3 binds to a novel short splice isoform of the transcription factor PAX6, and overexpression of PAX6 results in the translocation of β_3 to the nucleus, where β_3 is able to regulate the transcriptional function of PAX6 (REF. 139). Thus, several different studies have revealed that β_3 and β_4 have roles in gene transcription.

Conclusions and perspectives

In this Review, I have summarized findings from many groups that together indicate that both the $\alpha_2\delta$ and β subunits have major and complementary roles in the trafficking and stability of VGCCs, as well as in influencing their biophysical properties (FIG. 6). In different ways, $\alpha_2\delta$ and β subunits influence the microdomain localization of the channel complexes by forming a bridge to other proteins and by associating with cholesterol-rich membrane compartments. However, these auxiliary subunits may also be present in subcellular compartments separately from calcium channels, and have been found to interact with other proteins, including PrP and thrombospondin. It has yet to be confirmed whether these interactions are direct, and their physiological relevance remains to be fully established. It is therefore quite feasible that $\alpha_2\delta$ and β subunits may exhibit functions that are independent of calcium channels. Some of these independent functions have been described here, and it is highly likely that in the future, additional functions of these proteins will be elucidated.

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Competing interests statement
The author declares competing financial interests; see Web version for details.

FURTHER INFORMATION
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The 3DLigandSite website: http://www.sbg.bio.ic.ac.uk/3dligandsite
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