Chapter 2
Neuronal Functions of Auxiliary Calcium Channel Subunits

Gerald J. Obermair and Bernhard E. Flucher

Abstract In the central nervous system the second messenger calcium regulates neurotransmitter release, gene regulation, and neuronal plasticity. Voltage-gated calcium channels provide the major regulated calcium entry pathway in the membrane of neurons. They operate in a heteromultimeric complex between a pore forming α_1, and the auxiliary β and $\alpha_2\delta$ subunits. The cytoplasmic β and the extracellular membrane-attached $\alpha_2\delta$ subunit are required for the proper functional expression of the entire calcium channel complex. Moreover, the auxiliary subunits modulate the gating properties of the calcium channel and serve as scaffolds for upstream regulators and downstream effectors. Any of these properties affect the size of the calcium signal and in the synapse lead to changes in the functional coupling to neurotransmitter release. Beyond their classical role as auxiliary calcium channel subunits, β and $\alpha_2\delta$ have recently been implicated in cellular and neuronal functions independent of the channel complex. Here we review the experimental evidence pertinent to the many facets of auxiliary calcium channel function. We extract from it common principles and attempt to depict the state of the art of their role in regulating presynaptic function.

Keywords Voltage-gated calcium channels • Synaptic transmission • $\alpha_2\delta$ • β • High-voltage activated Ca^{2+} channels • Channel trafficking

2.1 Introduction

In excitable cells voltage-gated calcium channels (Ca_Vs; also termed voltage-dependent or voltage-operated calcium channels) mediate and regulate a variety of functions ranging from muscle contraction, secretion, synaptic function to gene

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regulation. Calcium entering through voltage-gated calcium channels operates as a local second messenger by activating downstream signalling proteins localized in the close vicinity of the channel pore. In neurons Ca_Vs contribute to the specific action potential firing pattern, presynaptic Ca_Vs regulate neurotransmitter release (Stanley 1993) and postsynaptic Ca_Vs are involved in the transcriptional regulation of CREB (cAMP-responsive element-binding protein) and NFAT (nuclear factor of activated T cells) (Deisseroth et al. 2003; Dolmetsch 2003) and thus likely play a crucial part in the formation of new memory (Moosmang et al. 2005). Over the recent years a detailed picture on the distribution and function of pre- and postsynaptic calcium channel types has begun to emerge. Thus, in the central nervous system Ca_Vs of the Ca_V2 family, namely P/Q-type ($Ca_V2.1$), N-type ($Ca_V2.2$), and R-type ($Ca_V2.3$) channels, are the major presynaptic pore forming subunits triggering synaptic release. The L-type channels $Ca_V1.2$ and $Ca_V1.3$ are mainly involved in postsynaptic functions including plasticity and gene transcription. The importance of the pre- and postsynaptic Ca_V pore-forming subunits is emphasized by the existence of channelopathies caused by loss-of-function as well as gain-of-function mutations (Pietrobon 2010; Striessnig et al. 2010). For example, dysregulation of presynaptic P/Q-type and postsynaptic L-type channels is involved in the etiology of migraine (Pietrobon 2010) and autism disorders (Splawski et al. 2004), respectively. In contrast, there is little to no evidence for a function of the skeletal muscle $Ca_V1.1$ isoform in the nervous system (Sinnegger-Brauns et al. 2009). The $Ca_V1.4$ isoform appears to be specifically expressed in the retina and its mutation causes congenital stationary night blindness type 2 (Wycisk et al. 2006a, b). Low-voltage activated calcium channels (T-type channels) $Ca_V3.1$, 3.2 and 3.3 are critical regulators of neuronal excitability. They are prominently expressed both in the central and peripheral nervous system and are involved in neurological disorders such as absence epilepsy and neuropathic pain (Iftinca 2011).

Ca_Vs operate in heteromultimeric complexes with the auxiliary β (also termed $Ca_V\beta$) and $\alpha_2\delta$ subunits, calmodulin and other calcium binding and regulating proteins. The pore-forming α_1 subunit of voltage-gated calcium channels defines the basic biophysical, pharmacological and physiological properties of the channels. A plethora of studies within the last 20 years have extensively demonstrated their roles in the localization, trafficking and stabilization of the channel complex (reviewed in Arikkath and Campbell 2003; Obermair et al. 2008; Dolphin 2009; Buraei and Yang 2010). The great majority of these studies was performed with different channel subunit combinations heterologously expressed in Xenopus laevis oocytes or mammalian expression systems such as human embryonic kidney (HEK) cells. Therefore the informative value of these studies regarding the role of the auxiliary calcium channel subunits in native cell systems like neurons remained limited. Whereas studies in heterologous expression systems are ideally suited to investigate effects and mechanisms for the interaction of specific coexpressed subunit partners in isolation, such studies do not predict as to whether the same protein-protein interactions indeed occur in signaling complexes of differentiated cells. Neither can it be assumed that in the complex with additional up- and downstream interacting proteins in differentiated cells the properties and effects

of such interactions are the same as in heterologous expression systems. The development of powerful neuronal expression systems and the analysis of calcium channel knock-out animal models (see box) in recent years have helped to reveal the physiological importance of auxiliary β and $\alpha_2\delta$ subunits in neuronal/synaptic function. With respect to the role of auxiliary calcium channel subunits in synaptic function the principal questions that now can be addressed include:

- What is the complement of specific calcium channel isoforms expressed in synaptic compartments?
- Do different subunit isoforms serve distinct functions and to what degree can they be compensated by other isoforms?
- Do the auxiliary subunits exclusively function in the context of the calcium channel (i.e., regulate its expression and targeting, or modulate its gating properties) or do auxiliary calcium channel subunits also serve functions independent of the channel?

2.2 Structure and Function of Auxiliary Calcium Channel Subunits

2.2.1 The $\alpha_2\delta$ Subunit

A total of four genes (Cacna2d1-4) encode for $\alpha_2\delta$ subunits ($\alpha_2\delta$-1 to $\alpha_2\delta$-4), which display distinct tissue distribution and out of which three isoforms ($\alpha_2\delta$-1 to -3) are strongly expressed in the central nervous system (CNS) (Arikkath and Campbell 2003; Schlick et al. 2010). $\alpha_2\delta$-1 and $\alpha_2\delta$-2 subunits are the primary targets for the anti-epileptic and anti-allodynic drugs gabapentin (GBP) and pregabalin (PG), which have also proven clinical efficacy in the treatment of generalized anxiety disorders (Bryans and Wustrow 1999; Rickels et al. 2005). Mature $\alpha_2\delta$ subunits consist of posttranslationally cleaved α_2 and δ peptides, which are associated to each other by a disulfide bond (Calderon-Rivera et al. 2012). Until recently it had been suggested that the δ subunit constitutes a single-pass membrane protein, and the α_2 subunit a highly glycosylated extracellular protein. However, this classical view has recently been challenged by the observation that $\alpha_2\delta$ subunits can form GPI-anchored proteins and that this posttranslational modification may be crucial for $\alpha_2\delta$ function (Davies et al. 2010). In either way the vast majority of the $\alpha_2\delta$ protein is extracellular, ideally situated to interact with constituents of the extracellular matrix or extracellularly exposed proteins. Consistent with a role in extracellular signaling is the domain structure of α_2. A von Willebrand factor type A (VWA) domain and two Cache domains were identified by sequence homology in all $\alpha_2\delta$ subunits (Anantharaman and Aravind 2000; Canti et al. 2005; Davies et al. 2007). VWA-domains are found in a variety of extracellular matrix proteins and integrin receptors and are well known for their role in cell-cell adhesion (Whittaker and Hynes 2002) involving a metal ion-dependent adhesion site (MIDAS). The integrity

of the MIDAS motif in $\alpha_2\delta$-2 has been shown to be necessary for calcium current enhancement and Ca_V channel trafficking (Canti et al. 2005). Cache domains were named after their presence in calcium channels and chemotaxis receptors and have been suggested to be involved in small molecule interactions (Anantharaman and Aravind 2000). Thus, it has been hypothesized that these domains may be regulated by small endogenous ligands, such as the amino acid isoleucine (reviewed in Dooley et al. 2007), and that they are involved in GBP and PG binding (Davies et al. 2007). $\alpha_2\delta$ subunits also contain a conserved N-terminal α-helical domain found in several methyl-accepting chemotactic receptors and mutations within this domain have been shown to interfere with GBP and PG binding (Anantharaman and Aravind 2000).

2.2.2 The β Subunit

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

have not been successful. However, the high efficacy of members of the small G-protein Rem/Gem/Kir family in blocking calcium currents by interacting with the β subunit holds great promise for these calcium channel subunits as drug targets (Yang et al. 2007).

2.3 Neuronal Voltage-Gated Calcium Channel Complexes

In differentiated cells calcium channels do not function in isolation, rather they exert their functions in the context of multimolecular signalling complexes. The short range of the second messenger calcium necessitates that downstream signalling proteins and effectors are anchored in the close vicinity of the channel pore. Similarly, increasing evidence shows that upstream modulators, like protein kinases and phosphatases achieve substrate specificity and increased signalling efficiency if they pre-exist in a complex with the channel. Accordingly, a voltage-gated calcium channel complex is composed of the calcium channel subunits proper, upstream modulators and downstream effectors, and the adapter and scaffold proteins, assembling the complex.

In neurons two such complexes have been subject to extensive investigations: First, the synaptic vesicle fusion apparatus and second, the postsynaptic calcium channel complex mediating excitation-transcription coupling.

The presynaptic calcium channel complex: Calcium influx through Ca_Vs transduces membrane depolarization into the chemical signal triggering fusion of neurotransmitter vesicles. Here Ca_Vs of the Ca_V2 subclass (P/Q- and N-type) associate with the SNARE proteins of the synaptic core complex either directly by an interaction of the SYNPRINT domain within the II-III loop of the α_1 subunits with syntaxin, SNAP-25 and synaptotagmin-1 (reviewed in Sheng et al. 1998; Zamponi 2003; Catterall 2011), or via the β subunit and the Rab interacting protein (RIM) (Kiyonaka et al. 2007). These interactions are believed to anchor the Ca_V channel close to the calcium sensor synaptotagmin and conversely to functionally modulate the calcium current, both enhancing the channel's efficacy to activate vesicle fusion. Indeed a low number of channels and in the extreme even a single channel opening is sufficient for triggering vesicle fusion (Stanley 1993; Bucurenciu et al. 2010). Neurotransmitter release is commonly modulated by neuropeptides and hormones. Therefore G-protein coupled receptors (GPCRs), G-proteins, phospholipases, adenylate cyclases, and protein kinases may coexist with presynaptic calcium channel complexes.

The postsynaptic calcium channel complex: The postsynaptic Ca_V complex mediates excitation-transcription coupling. Here activation of L-type channels (Ca_V1) initiates a signalling cascade to the nucleus that regulates gene expression. To this end scaffold proteins like AKAP79/150 recruit protein kinases and the calcium/calmodulin dependent protein phosphatase calcineurin to the channel.

Upon activation by the local calcium signal this signalling cascade leads to the translocation of NFATc4 into the nucleus and (Oliveria et al. 2007; Ma et al. 2011). Since overexpression of AKAP79/150 also enhances the L-type calcium currents (Altier et al. 2002) it seems that at least some of these signalling proteins are shared with upstream signalling cascades mediating GPCR-induced phosphorylation of the channel. Indeed β adrenergic receptors, AKAP, PKA and calcineurin were all detected in the $Ca_V1.2$ signaling complex in neurons (Davare et al. 2001; Dai et al. 2009).

The subunit composition of the pre- and postsynaptic Ca_Vs is expected to influence the function of these signalling complexes in several ways. Modulation of current properties by auxiliary subunits will affect the signalling power of the complex. A participation of the auxiliary calcium channel subunits in scaffolding will affect the composition of the signalling complex and thus the signalling specificity. By targeting the channel into the close proximity of effector proteins the efficacy of the signalling process will be enhanced. As different subunits differ with respect to their modulatory properties, protein-protein interactions and subcellular targeting, as well as the molecular diversity of the auxiliary subunits may be important for the proper assembly and function of the different calcium channel complexes in neurons. In other words, the distinct molecular organizations and functions of different calcium channel signalling complexes may require a specific subunit composition of the channel. In turn, the distinct molecular compositions of pre- and postsynaptic signalling complexes may favour the incorporation of channels with specific subunit compositions.

2.3.1 Potential Mechanisms for Establishing Specific Neuronal Ca_V Complexes

Understanding the specificity of Ca_V subunit interactions in native differentiated cell systems is key for resolving the physiological neuronal functions of calcium channels and their auxiliary subunits. Three distinct mechanisms may explain the assembly of specific $\alpha_1/\beta/\alpha_2\delta$ subunit complexes in neurons (see Fig. 2.1):

1. Different affinities of auxiliary β and $\alpha_2\delta$ subunits for specific α_1 isoforms may favour the preferential association of specific subunit combinations.
2. Limiting the number of isoforms expressed in a specific cell type at a given time will also favour the formation of a specific Ca_V complex.
3. Distinct subcellular targeting properties of individual subunits as well as protein-protein interactions with other proteins may yield complex specificity.

Alternatively, specific stable complexes may not exist in all neuronal compartments and Ca_V channels could be regulated by reversible interactions with pools of functionally distinct cytoplasmic β subunits or membrane anchored $\alpha_2\delta$ subunits.

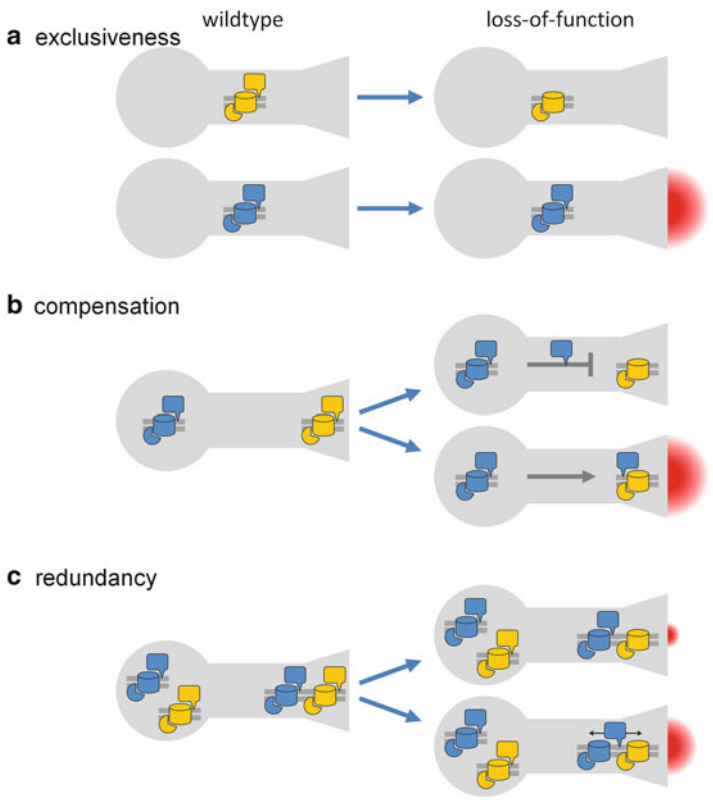

Fig. 2.1 Model explaining loss-of-function scenarios of distinct mechanisms for the assembly of specific $\alpha_1/\beta/\alpha_2\delta$ subunit complexes in neurons. (**a**) Different cell types may express exclusive $\alpha_1/\beta/\alpha_2\delta$ complexes (either *orange* or *blue*). Knockout of one subunit (*orange* $\alpha_2\delta$) will lead to a loss of function (e.g. synaptic function) in one cell type, whereas the functions of other cell types are not affected. (**b**) Specific $\alpha_1/\beta/\alpha_2\delta$ complexes may form by differential targeting into distinct subcellular compartments (soma-*blue*, synapse-*orange*). The consequence of knockdown of one subunit (*orange* $\alpha_2\delta$) depends on the uniqueness of the targeting properties of the remaining isoforms. Thus, if axonal targeting of the blue isoform is not possible, knockout will ultimately lead to a loss of function (*upper*). Otherwise the blue isoform may compensate for the loss of the orange isoform and restore normal synaptic function (*lower*). (**c**) Specificity of $\alpha_1/\beta/\alpha_2\delta$ complexes may be determined by distinct affinities or distinct interaction partners in a macromolecular complex. Putative compensatory mechanisms will then depend on the stringency of the individual complexes. If subunit exchange between the individual complexes is excluded, functional compensation is limited (*upper*). If subunits can associate/dissociate with and from individual complexes, at least partial compensation will occur (*lower*)

2.3.1.1 The Importance of Affinity for Specific Ca_V Complex Formation

Heterologous coexpression studies have demonstrated that all four β subunits as well as all four $\alpha_2\delta$ subunits can enhance the trafficking and modulate the current properties of all high-voltage activated calcium channel α_1 subunits, indicating a

great promiscuity of subunit interactions (reviewed in Arikkath and Campbell 2003; Dolphin 2003; Obermair et al. 2008; Buraei and Yang 2010). In line with this observation low neuronal α_1-β selectivity was suggested by immunoprecipitation experiments showing similar β subunit compositions of neuronal L-type, P/Q-type and N-type channels (Liu et al. 1996; Scott et al. 1996; Pichler et al. 1997). Furthermore, biochemical analysis revealed similarly high affinities of different β subunits to the AID (De Waard et al. 1995; Van Petegem et al. 2008). Therefore, if differences in the strength of interactions contribute to the formation of preferential subunit compositions, these may be determined by low affinity secondary interaction sites rather than by the AID, and/or by indirect interactions involving additional components of the signalling complex. In fact, recent experiments in skeletal muscle indicate that, although non-muscle β subunits can successfully compete with the skeletal muscle β_{1a} for association with the channel, complexes including the heterologous isoforms are less stable than those consisting of all skeletal muscle isoforms (Campiglio et al. 2013). These experiments for the first time demonstrate the formation of complexes with preferential subunit composition in a native calcium channel complex, and suggest that isoform specific differences in the association with the α_1 subunit underlie the distinct complex stabilities. For $\alpha_2\delta$ subunits no high-affinity binding sites have been identified in the α_1 subunit. Interestingly, although in the initial biochemical purification of neuronal Ca_V channels $\alpha_2\delta$ reliably co-purified with the α_1 and β subunits (McEnery et al. 1991; Witcher et al. 1994; Martin-Moutot et al. 1995; Liu et al. 1996), a recent quantitative proteomics approach of mammalian Ca_V2 channel complexes in brain extracts did not identify $\alpha_2\delta$ subunits as a core components of the complex (Müller et al. 2010).

Also upon coexpression in nerve or muscle cells, $\alpha_2\delta$ subunits appear more widely expressed throughout the plasma membrane than the other channel subunits (Schredelseker et al. 2005) (Schöpf, Obermair et al., unpublished observation). If distinct affinities of auxiliary subunit isoforms to preferential α_1 subunit partners contribute to the formation of specific complexes, this may involve low-affinity binding sites, which so far eluded biochemical detection. Such low affinity interaction would allow dynamic exchange of subunits in response to changes in expression levels or local concentration of the auxiliary subunits.

2.3.1.2 Spatial and Temporal Separation of Ca_V Subunit Expression

Distinct cellular expression levels of calcium channel isoforms indeed provide an important determinant of complex specificity. They have been identified in non-neuronal cells such as skeletal muscle ($Ca_V1.1/\beta_{1a}/\alpha_2\delta$-1) and cardiac myocytes ($Ca_V1.2/\beta_2/\alpha_2\delta$-1) (Arikkath and Campbell 2003) and specialized neuronal cell types like retina photoreceptor cells ($Ca_V1.4/\beta_2/\alpha_2\delta$-4) (Ball et al. 2002; Barnes and Kelly 2002; Wycisk et al. 2006a; Neef et al. 2009). Similarly, the cerebellum shows a strong preference towards expression of one subunit combination ($Ca_V2.1/\beta_4/\alpha_2\delta$-2) (Ludwig et al. 1997; Brodbeck et al. 2002). We have recently shown that murine

cortex, hippocampus and cerebellum simultaneously express mRNA of five out of seven high-voltage activated α_1 subunits, all four β subunit isoforms, and three of four $\alpha_2\delta$ subunits at physiologically relevant levels (Schlick et al. 2010). Surprising was our finding that also a single cell type, cultured hippocampal pyramidal cells expresses all the same Ca_V subunit isoforms as hippocampus. This clearly suggests that in cultured hippocampal pyramidal cells, a restricted expression of auxiliary subunit isoforms is not the strategy to achieve specific Ca_V subunit compositions. Consequently other mechanisms, like specific targeting properties and interactions with anchoring proteins in pre- and postsynaptic compartments, must be responsible for assembling channels with distinct subunit compositions.

In neurons expression patterns of channels and signaling proteins are not static. Many receptors, channels and transport proteins serve different functions or have different properties during distinct phases in the life cycle of a neuron. Consequently their expression patterns change during development (Schlick et al. 2010) and in some cases they undergo an isoform switch during differentiation (Vance et al. 1998). During development Ca_Vs serve in the regulation of neuronal mobility, path-finding and synapse formation (Pravettoni et al. 2000; Zheng and Poo 2007). These functions likely require different subunit isoforms or splice variants than in differentiated neurons. Moreover, neurons possess the unique ability to alter expression and composition of synaptic proteins in an activity-dependent manner. It can be expected that any of these changes also go hand in hand with altered subunit compositions or splice variant expression of Ca_Vs. At present hardly any information on the differential expression and subunit composition of Ca_Vs during neurogenesis and synaptic plasticity is available. Once the tools for analyzing expression patterns in specific populations of neurons are in place, the study of changing channel subunit combinations during differentiation and synaptic plasticity will be an important and fruitful undertaking.

2.3.1.3 Differential Targeting and Localization of Auxiliary Ca_V Subunits

Neurons are compartmentalized and structurally and functionally polarized more than any other cell type. Accordingly the composition of membrane proteins differs greatly between the input side, the somato-dendritic compartment, and the output side, the axonal compartment. Moreover, pre- and postsynaptic membranes differ from extrasynaptic membrane domains in composition of the lipid and protein content. Such compartmentalization requires complex targeting mechanism for most of the membrane proteins. However, Ca_Vs serve important functions in both the pre- and postsynaptic compartment. Whereas the functional expression and targeting of calcium channels is unique in each neuronal cell type, an overall preference exists of Ca_V2 and Ca_V1 channels for the pre- and postsynaptic compartment, respectively. The specific interaction of Ca_V2 channels with the presynaptic fusion apparatus has been shown to contribute to this differential targeting (Mochida et al. 2003; Szabo et al. 2006; Simms and Zamponi 2012). It can be expected that the auxiliary calcium channel subunits also display differential targeting properties in neurons. If they

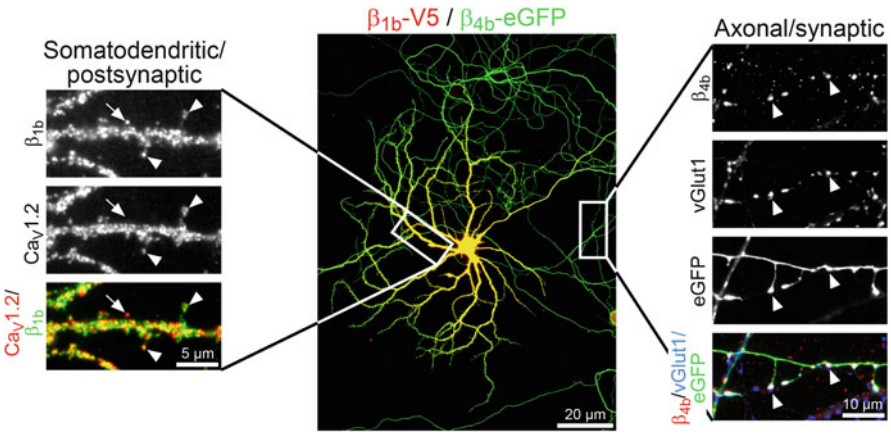

Fig. 2.2 Isoform-specific localization of V5-tagged β subunits in cultured hippocampal neurons. *Center*: A 17 DIV cultured hippocampal neuron cotransfected with a V5-tagged β_{1b} and an eGFP-tagged β_{4b} subunit. The distribution of β_{1b}-V5 is confined to the somatodendritic compartment (*yellow neurites*) whereas β_{4b}-eGFP expression is high throughout the axon and the axonal branches (*green neurites*). *Left*: V5-tagged β_{1b} co-localizes with membrane expressed HA-tagged $Ca_V1.2$ channel clusters along the dendrites and in dendritic spines (*arrowheads*). Note that some β_{1b} clusters (*arrow*) do not colocalized with $Ca_V1.2$, indicative of an association with a different Ca_V α_1 subunit. *Right*: Example of the presynaptic localization of a β subunit in a triple-labelling experiment. Fluorescence of soluble eGFP allows to morphologically identify axons with their short axonal branches with presynaptic terminals identified by staining for the vesicular glutamate transporter (vGlut1). The V5-tagged β_{4b} isoform specifically accumulates in presynaptic terminals (examples indicated by *arrowheads*). For more details see Obermair et al. (2010)

formed preferential complexes with specific α_1 subunits, their targeting mechanisms would drag the auxiliary subunits along. $\alpha_2\delta$ and β subunits would not require independent targeting mechanisms. However, if the auxiliary subunits possessed targeting mechanisms independent of α_1 subunits, they could contribute to the differential distribution of the channels in the pre- and postsynaptic compartments.

Previously we have employed the expression of epitope-tagged β subunits and subsequent immunofluorescence labeling with a single antibody as a powerful approach to analyze their targeting behavior (Obermair et al. 2010). On the one hand all four examined β subunit isoforms and two β_1 and β_2 splice variants were found in the somato-dendritic as well as in the axonal compartment. Importantly, the β_1 splice variants were less efficiently targeted to the distal axon, indicating a preferential role of in the postsynaptic/somatodendritic compartment (Fig. 2.2). However, even though the β_1 isoform was poorly targeted to the distal axon, it could be incorporated into the nerve terminal like β_2, β_3, and β_4. This observation is consistent with the great promiscuity of α_1/β interactions observed upon heterologous coexpression and indicates that in neurons the affinities of specific β-AID pairs to each other (De Waard et al. 1995) by themselves do not determine the specificity of α_1/β assemblies.

The great promiscuity in the interaction of β subunits with distinct α_1 subunits allows for the differential modulation of Ca_Vs by the association and dissociation with different β subunits (Obermair et al. 2008). Such a dynamic exchange of neuronal β isoforms with Ca_V1 channels has recently been demonstrated in differentiated skeletal myotubes (Campiglio et al. 2013). Thus, in neurons which express all β isoforms, shifts in the relative expression or local concentration of functionally distinct channel subunits may change the equilibrium between the subunit partners and thus the subunit composition of the channel complexes.

Neuronal $\alpha_2\delta$ subunits display regional differences in their expression levels in brain (Cole et al. 2005; Taylor and Garrido 2008). Nevertheless, similar to β subunits three out of four $\alpha_2\delta$ subunits are simultaneously expressed in neurons of the CNS (Schlick et al. 2010; Nimmervoll et al. submitted). $\alpha_2\delta$ subunits are abundantly expressed in the neuronal plasma membrane (Bauer et al. 2009; Müller et al. 2010) and one common feature is their localization in presynaptic terminals. Accordingly $\alpha_2\delta$-1 to -3 isoforms localize to synapses upon overexpression in hippocampal neurons and can interact with presynaptic calcium channels (Hoppa et al. 2012; Nimmervoll et al. submitted). In the hippocampus immunostaining with a monoclonal antibody revealed a preferential localization of $\alpha_2\delta$-1 in mossy fibre terminals in the CA3 region of the hippocampus (Taylor and Garrido 2008). Recently characterized $\alpha_2\delta$-3 mutants in C. elegans (*unc-36*) and Drosophila (*straightjacket*) suggest a primary role in presynaptic calcium channel trafficking (Dickman et al. 2008; Saheki and Bargmann 2009). In addition $\alpha_2\delta$ subunits are synaptically expressed in specialized nerve cells including retinal photoreceptor cells (Mercer et al. 2011), dorsal root ganglion neurons (Bauer et al. 2009) and in synapses along the hearing pathway (Pirone et al. 2009). Apart from presynaptic localizations in the cerebellum $\alpha_2\delta$-2 is concentrated in lipid rafts, suggestive of a restricted expression in microdomains, which may be important for its interaction with $Ca_V2.1$ channels (Davies et al. 2006). Nevertheless, it is currently not known whether $\alpha_2\delta$ subunits display an isoform specific targeting pattern and to which extent their localization depends on the interaction with α_1 subunits and vice versa.

2.4 Neuronal Functions Related to Auxiliary Calcium Channel Subunits

2.4.1 Channel Trafficking and Current Modulation by β Subunits

The auxiliary $\alpha_2\delta$ and β subunits are important factors for trafficking Ca_Vs to the plasma membrane and possibly for stabilizing them in functional signaling complexes. Because an increased density of functional channels in the synapse is expected to raise the efficacy of synaptic release, regulating membrane expression may act as an efficient mechanism to modulate synaptic function. Especially the

β subunit has long been known to strongly increase calcium current density upon coexpression in HEK cells or X. laevis oocytes (reviewed in Dolphin 2003; Buraei and Yang 2010). Recent studies provided insight into the role of β subunits in membrane targeting of calcium channels in native cells and tissues (Tab. 2.1; reviewed in Buraei and Yang 2010).

For example, antisense knockdown of β subunits in cultured rat dorsal root ganglion neurons strongly decreased barium currents through endogenous calcium channels (Berrow et al. 1995). We could recently show that mutation of the AID in $Ca_V1.2$ channels completely prevented the surface expression of $Ca_V1.2$ in cultured hippocampal neurons (Obermair et al. 2010). Furthermore overexpression of β subunits substantially increased surface expression of $Ca_V1.2$ channels, indicating that the abundance of β subunits may present a limiting factor for the membrane expression of Ca_Vs, and that the number of channels in the neuronal membrane can indeed be regulated by the amount of available β subunits.

The mechanism by which β subunits enhance membrane expression has long been a matter of discussion. Previously it has been suggested that β enables ER export of α_1 subunits by masking an ER retention signal within the I-II intracellular loop of Ca_V1 α_1 subunits (Bichet et al. 2000). Using an elegant combination of electrophysiology and quantification of channel surface expression in HEK cells, Fang and Colecraft have systematically characterized the contribution of all intracellular domains of $Ca_V1.2$ for the β-mediated surface expression (Fang and Colecraft 2011). These experiments clearly demonstrated that the I-II linker contains a putative ER export motif and that the β-dependent increase in surface expression may require a C-terminus-dependent rearrangement of intracellular domains, thereby overcoming retention signals within the other cytoplasmic loops. Furthermore, two recent studies demonstrated that association with a β subunit prevents the proteasomal degradation of the respective α_1 subunits, thereby stabilizing and increasing the surface expression (Altier et al. 2011; Waithe et al. 2011).

In addition to effects of β subunits on membrane targeting, β subunits are powerful modulators of the channel's gating properties. Upon coexpression in heterologous cells β subunits enhance the voltage-dependent activation and inactivation. The most notable isoform-specific effect is the strong inhibition of voltage-dependent inactivation by the palmitoylated β_{2a} (Qin et al. 1998).

There are multiple lines of evidence demonstrating that β subunits modulate calcium channel functions in neurons. For example overexpression of β_{2a} and β_{4b} in hippocampal neurons induce depression and paired-pulse-facilitation of autaptic synapses, most likely by a differential modulation of the current properties of presynaptic Ca_Vs (Xie et al. 2007). Moreover, Ca_V2 channels are subject to presynaptic inhibition by hormones and neurotransmitters through G-protein coupled receptors linked to $G_{i/o}$ via Gβγ. This inhibition, which may be involved in short-term synaptic plasticity, is voltage-dependent and depends on the presence of the β subunit in an isoform-specific manner (reviewed in Dolphin 2003). It appears that G-protein βγ association with Ca_V2 channels antagonizes the effects of the β subunit on voltage-dependent activation. The larger the hyperpolarizing effect of the

β subunit, the larger the G-protein induced inhibition. Conversely, the β subunits increase the dissociation of Gβγ and thus relieve inhibition during paired pulse facilitation (Canti et al. 2000; Feng et al. 2001).

A similarly strong β subunit dependence on GPCR modulation of Ca_Vs via Gq-proteins has been reported. Both potential mechanisms, inhibition of Ca_V channels by phosphatidylinositol 4,5-bisphosphate (PIP_2) depletion or arachidonic acid generation, are strongly abated upon coexpression of the palmitoylated β_{2a} isoform (Heneghan et al. 2009; Suh et al. 2012). Thus, lipid modulation together with the nature of the Ca_V-associated β subunit emerges as a powerful modulator of neuronal excitability or neurotransmitter release (Striessnig 2009).

The RGK (Rad, Rem, Rem2, Gem/Kir) family of small monomeric GTP-binding proteins are potent inhibitors of neuronal Ca_Vs; both when heterologously expressed and in native cells including neurons (Chen et al. 2005; reviewed in Buraei and Yang 2010). Multiple inhibitory mechanisms have been suggested including inhibition of membrane expression due to binding to and sequestration of the β subunit and current inhibition of channels preexisting in the membrane. Although calcium current inhibition by RGK proteins absolutely depends on the β subunit and its properties are reminiscent of Gβγ inhibition (see above), recent mutagenesis studies indicate that they use distinct mechanisms (Fan et al. 2010). Whether this potent inhibitory mechanism actually is in effect in synapses, and if so, how it would be activated in neurons remains to be investigated.

Ca_Vs can be regulated by phosphorylation of the α_1 subunits as well as the β subunits. PKA, PKC, CaMKII, PI3K/Akt and MAPK have all been shown to phosphorylate β subunits and modulate calcium currents in a β-dependent and isoform-specific manner (Dolphin 2003). For some of these protein kinases the phosphorylation sites in the β subunit have been identified and mutation thereof has been demonstrated to abolish the modulatory effects. If active in the synapse, any of these mechanisms might be fit to modulate synaptic transmission. Moreover, isoform-specific differences in phosphorylation add to the functional heterogeneity and potential specificity of modulatory mechanisms in synapses expressing channels of different subunit composition. However, whereas the physiological role of calcium channel phosphorylation in the fight-or-flight response is well established in the heart (Fuller et al. 2010), a similar role in presynaptic function, and particularly the involvement of β subunits is still elusive.

Ca_Vs functionally interact directly and indirectly via the β subunit with a number of other ion channels and signaling proteins including calcium-activated K^+ channels, bestrophin, the ryanodine receptor, dynamin, synaptotagmin I, and the Rab interacting protein RIM1. Most of these proteins can be found in synapses and therefore could potentially function as up- or downstream modulators of synaptic function. As of today the best candidate for such a modulation is RIM1, which is essential for synaptic transmission and plasticity and binds to β subunits with high affinity (Kiyonaka et al. 2007). This interaction appears to affect presynaptic function in two ways. First it is important for docking neurotransmitter vesicles to Ca_V2 channels, and secondly it modulates voltage-dependent inactivation of the

channel. In heterologous expression systems this interaction was observed with any of the β isoforms coexpressed with Ca_V2 channels. Whether in the context of the synapse the RIM1-β interaction displays more isoform specificity remains to be investigated.

2.4.2 *Channel Trafficking and Current Modulation by $\alpha_2\delta$ Subunits*

The roles of $\alpha_2\delta$ subunits in synaptic function are less well defined than those of the β subunits. When heterologously expressed all $\alpha_2\delta$ subunit isoforms can modulate the trafficking and/or the current properties of Ca_V α_1 subunits (reviewed in Arikkath and Campbell 2003; Davies et al. 2007; Obermair et al. 2008). In skeletal and cardiac muscle, for example, $\alpha_2\delta$-1 determines the typical current properties of the respective L-type Ca_Vs (Obermair et al. 2005, 2008; Tuluc et al. 2007; Gach et al. 2008). Therefore $\alpha_2\delta$-1 is an important determinant of action potential duration in cardiac myocytes (Tuluc et al. 2007; Templin et al. 2011). When coexpressed with neuronal P/Q- or N-type channels all three neuronal $\alpha_2\delta$ subunits cause an increase in current density (e.g., Davies et al. 2007). Conversely shRNA depletion of $\alpha_2\delta$-1 in the skeletal muscle expression system strongly reduced heterologously expressed $Ca_V2.1$ and $Ca_V2.2$ currents (Obermair et al. 2008).

Based on these results a role of $\alpha_2\delta$ subunits in triggering neurotransmitter release, which is directly related to the number of presynaptic Ca_Vs (Schweizer et al. 2012), was to be expected. Nevertheless, conflicting results have been reported on the effects of GBP administration on synaptic functions. Whereas acute application of these drugs has only mild effects (if any) on calcium currents (Alden and Garcia 2001; Kang et al. 2002; Micheva et al. 2006; Davies et al. 2007; Dooley et al. 2007) chronic application of GBP has been shown to reduce both native N-type and heterologously expressed P/Q-type calcium currents by about 50 % (Hendrich et al. 2008). Thus it is meanwhile well established that chronic GBP treatment interferes with calcium channel trafficking to the cell surface (Tran-Van-Minh and Dolphin 2010; Dolphin 2012). The importance of $\alpha_2\delta$ subunits in presynaptic functions (see Table 2.1 and Fig. 2.3) related to their role in Ca_V targeting is further supported by the upregulation of $\alpha_2\delta$ subunits in animal models of neuropathic pain (Bauer et al. 2009; Lu et al. 2010) and impaired Ca_V trafficking after chronic GBP and PG treatment (Bauer et al. 2009; Tran-Van-Minh and Dolphin 2010). Also the recently identified interaction of $\alpha_2\delta$-1 with mutant prion protein was shown to impair proper membrane trafficking of the calcium channel complex and consequently reduced glutamatergic transmission in CGNs (Senatore et al. 2012). Indeed chronic treatment with GBP significantly reduced synaptic release efficacy as measured by high KCl-induced FM-dye release in cultured hippocampal neurons (Nimmervoll et al. submitted). This GBP mediated inhibition of synaptic release was augmented in cultures from $\alpha_2\delta$-3 null neurons, indicating that $\alpha_2\delta$-3 partially compensated for the effects of GBP on $\alpha_2\delta$-1 and -2. A similarly strong effect of chronic application

Table 2.1 Effects of auxiliary calcium channel subunits on synaptic functions

	Basic modulatory effect	Mechanism (mediator)	Evidence[a]
β	Trafficking and membrane expression	Direct	Neef et al. (2009), Obermair et al. (2010), and Li et al. (2012)
		RGK GTPases	Correll et al. (2008) and Leyris et al. (2009)
		Aminopyridines	Wu et al. (2009)
	Modulation of the calcium currents	G-protein modulation	Feng et al. (2001), Dolphin (2003), and Heneghan et al. (2009)
		RIM	Kiyonaka et al. (2007), Gebhart et al. (2010), and Gandini and Felix (2012)
		RGK GTPases	Correll et al. (2008) and Leyris et al. (2009)
		PIP2	Correll et al. (2008) and Suh et al. (2012)
		Gating properties	Xie et al. (2007)
		Aminopyridines	Wu et al. (2009)
	Linking calcium channels to release sites	RIM	Kiyonaka et al. (2007) and Gandini et al. (2011)
		Synaptotagmin	Vendel et al. (2006)
$\alpha_2\delta$	Trafficking and membrane expression	Direct	Dickman et al. (2008), Hendrich et al. (2008), Ly et al. (2008), Bauer et al. (2009), Saheki and Bargmann (2009) Martinez-Hernandez et al. (2011), Hendrich et al. (2012), Hoppa et al. (2012), and Nimmervoll et al. (submitted)
		PrP interaction	Senatore et al. (2012)
	Synapse formation/structural organization	Not known	Wycisk et al. (2006a)
		Not known	Kurshan et al. (2009)
		Thrombospondin	Eroglu et al. (2009)
		Vesicular signaling	Alix et al. (2008)
	Linking calcium channels to release sites	Not known	Hoppa et al. (2012) and Nimmervoll et al. (submitted)

[a]Including some indirect evidence for synaptic function

of PG on synaptic transmission between dorsal root ganglion and dorsal horn neurons, which primarily express $\alpha_2\delta$-1, has been observed (Hendrich et al. 2012).

shRNA knockdown of $\alpha_2\delta$-1 in hippocampal neurons reduced presynaptic expression of $Ca_V2.1$ and concomitantly synaptic release probability induced by single action potentials (Hoppa et al. 2012). Conversely, $\alpha_2\delta$ subunit overexpression increased presynaptic calcium channel density and release probability. However, at the same time the presynaptic calcium signal was significantly reduced. This suggests that $\alpha_2\delta$ subunits may be involved in linking presynaptic Ca_Vs to the release site. Surprisingly and in contrast to increased release probability upon

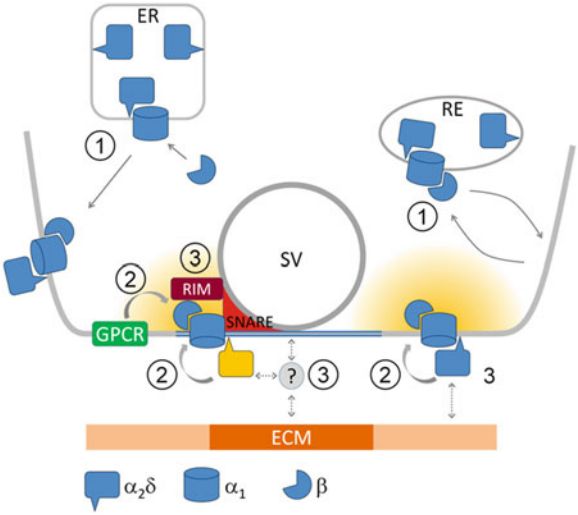

Fig. 2.3 Model summarizing the putative effects of the auxiliary $\alpha_2\delta$ and β subunits in the presynaptic compartment: (1) Trafficking: export from the endoplasmic reticulum (ER) by β; trafficking from recycling endosomes (RE) by $\alpha_2\delta$. (2) Ca_V current modulation: modulation by distinct β isoforms either directly, or by mediating modulatory mechanism in a β-isoform dependent manner (e.g. GPCR modulation); modulation by distinct $\alpha_2\delta$ subunits (indicated in *blue/orange*) by association/dissociation. (3) Linking calcium channels to the release site (*SV*, synaptic vesicle): β subunits via binding to RIM and SNARE proteins; $\alpha_2\delta$ subunits by association with a potential extracellular ligand and/or the extracellular matrix (*ECM*). Thereby individual $\alpha_2\delta$ isoforms (indicated in *orange*) may link calcium channels better to the release site by interaction with special ECM components or a specialized lipid domain in the synaptic membrane (indicated by *blue double line*)

single action potentials, in our own experiments we observed a slight reduction of synaptic FM-dye release during sustained depolarization upon $\alpha_2\delta$-1 overexpression (Nimmervoll et al. submitted). Thus, it is possible that $\alpha_2\delta$-1 overexpression one the one hand inhibits calcium influx and consequently synaptic release during prolonged depolarization like trains of action potentials or high KCl. On the other hand this might increase release probability due to a tighter association of calcium channels with the releases site upon single action potentials (Hoppa et al. 2012). Alternatively the reduction in the presynaptic calcium influx may be a consequence of a reduction of the action potential duration upon $\alpha_2\delta$ subunit overexpression (Hoppa et al. 2012). Thus, besides regulating synaptic transmission, $\alpha_2\delta$ subunits may control neuronal excitability, for example by increasing somatodendritic calcium channels and thus the coupling to calcium-activated potassium channels as previously characterized for BK channels (Berkefeld et al. 2006). Apart from regulating surface expression the specific functions of $\alpha_2\delta$ subunits on somatodendritic calcium channels have so far not been studied. While chronic treatment with GBP strongly reduced synaptic FM-dye release (see above), we found release kinetics to be unaffected in a double $\alpha_2\delta$-1 knockdown/$\alpha_2\delta$-3 knockout model. This strongly implicated the remaining

$\alpha_2\delta$-2 subunit to compensate for the loss of $\alpha_2\delta$-1 or $\alpha_2\delta$-3 dependent trafficking and modulation functions. However, it may also suggest that $\alpha_2\delta$-2 is chiefly involved in regulating transmitter release, likely by an association with presynaptic P/Q-type channels, which solely determine the kinetic properties of KCl-dependent FM-dye release in cultured hippocampal neurons (Nimmervoll et al. submitted).

A preferential correlation of $Ca_V2.1$ and $\alpha_2\delta$-2 expression was observed when quantifying overall Ca_V mRNA abundance (Schlick et al. 2010). Importantly, these recent findings on the differential synaptic roles of $\alpha_2\delta$ isoforms are further in agreement with the phenotypes of isoform specific $\alpha_2\delta$ subunit null mice. While $\alpha_2\delta$-1 and $\alpha_2\delta$-3 knockout mice display only mild overall CNS phenotypes (Fuller-Bicer et al. 2009; Neely et al. 2010), $\alpha_2\delta$-2 knockout or mutant (*ducky*, see box) mice display epilepsy and ataxia and show severely impaired cerebellar development (Brodbeck et al. 2002; Ivanov et al. 2004).

The altered release probabilities observed in hippocampal neurons from double knockout/knockdown cultures provided the first indirect evidence, that in a presynaptic bouton, which simultaneously expresses three $\alpha_2\delta$ isoforms, a single $\alpha_2\delta$ isoform may preferentially associate with a specific Ca_V α_1 subunit partner. However, it did not allow any conclusion on the nature or stability of this interaction and many studies described above favor a nonselective interaction of α_1 and $\alpha_2\delta$ subunits, similar to the promiscuity of the interaction with β subunits. In mouse chromaffin cells, for example, PG treatment blocked exocytosis by non-selectively inhibiting Ca_V1, $Ca_V2.1$ and $Ca_V2.2$ channels (Hernandez-Vivanco et al. 2012), further indicating the interaction of $\alpha_2\delta$-1 with distinct α_1 subunits. In agreement with these observations both indirect and direct evidence accumulated over the last years suggesting that $\alpha_2\delta$ subunits may not be tightly associated with channel complexes and also exist independent of the complex. In our own studies in skeletal muscle cells we could show that free $\alpha_2\delta$ exists in the plasma membrane without α_1 subunits, and that membrane expression of $\alpha_2\delta$ subunits appears to be independently regulated (Flucher et al. 1991; Obermair et al. 2005, 2008; Schredelseker et al. 2005). Similarly, not all cerebellar $Ca_V2.1$ α_1 subunits seem to be associated with an $\alpha_2\delta$-2 subunit (Davies et al. 2006). As mentioned above, proteomics of mammalian Ca_V2 channels did not identify $\alpha_2\delta$ subunits as core components of the complex (Müller et al. 2010). Thus, with the exception of cellular model systems that express an exclusive or at least preferential set of Ca_V α_1, β and $\alpha_2\delta$ subunits, little information exists on which and how $\alpha_2\delta$ subunits interact with the Ca_V complex.

2.4.3 Functions Independent of the Calcium Channel Complex

2.4.3.1 $\alpha_2\delta$ Subunits and Synapse Formation

Traditionally the auxiliary Ca_V subunits $\alpha_2\delta$ and β have been envisioned as stable components of the Ca_V complex in a 1:1 ratio with α_1 subunits. However, recently experimental evidence accumulated that suggests cellular function of these two

proteins that are in part or entirely independent of the Ca_V complex. As for the $\alpha_2\delta$ subunits several studies point towards a major calcium channel independent contribution to synapse formation, likely by interaction with components of the extracellular matrix (Fig. 2.3). $\alpha_2\delta$-1 has been shown to act as a receptor for thrombospondin, an astrocyte-secreted protein that promotes CNS snaptogenesis (Eroglu et al. 2009). Overexpression of $\alpha_2\delta$-1 strongly promoted, and shRNA knockdown inhibited excitatory synapse formation in cultured retinal ganglion cells. GBP treatment also inhibited synapse formation and the mechanism was shown to involve the $\alpha_2\delta$-1 VWF domain. Using a forward genetic screen, Drosophila mutants for the $\alpha_2\delta$-3 (*straightjackt; stj*) isoform have been identified which show defects in presynaptic Ca_V localization and synaptic function (Dickman et al. 2008). By further analyzing the phenotypes of the $\alpha_2\delta$-3 (*stj*) null mutants, it became evident that motoneurons failed to develop normal synapses (Kurshan et al. 2009). Interestingly, this phenotype was independent of the Drosophila pore forming α_1 subunit (*cacophony*) since *cacophony* null mutants showed no defect in synapse formation. Mutant (*du, du^{2J}, entla*) and targeted knockout mice for $\alpha_2\delta$-2 display altered morphology and reduced calcium currents in Purkinje cells (Barclay et al. 2001; Brill et al. 2004; Ivanov et al. 2004; Donato et al. 2006), also suggesting a defect in synapse formation. Finally, it has been shown that the spontaneous mouse mutant of $\alpha_2\delta$-4 (Cacna2d4) causes structural and functional abnormalities of retinal ribbon synapses associated with the loss of rods (Wycisk et al. 2006a).

All these strong effects of loss-of-$\alpha_2\delta$-function on synapse structure and formation where revealed in model systems that primarily express only one $\alpha_2\delta$ isoform, such as retinal ganglion cells, Drosophila motoneurons, and mammalian photoreceptors. In cellular systems which express more than one $\alpha_2\delta$ isoform, such as CNS neurons, both calcium channel dependent and independent functions of $\alpha_2\delta$ subunits appear to be subject to compensation by other $\alpha_2\delta$ isoforms. Recently we analyzed the density of functional synapses of $\alpha_2\delta$ loss-of-function models (Nimmervoll et al. submitted). We found that synapse formation was still close to normal in $\alpha_2\delta$-3 deficient cultured hippocampal neurons in which $\alpha_2\delta$-1 was knocked down or $\alpha_2\delta$-1 and $\alpha_2\delta$-2 were chronically blocked with GBP. Thus, it seems that the contribution of individual $\alpha_2\delta$ isoforms to synapse formation is limited in neurons expressing three different $\alpha_2\delta$ isoforms. This is further supported by the recent characterization of $\alpha_2\delta$-3 knockout mice, which did not reveal overall effects on synapse formation (Neely et al. 2010). To answer this question, it will ultimately be necessary to study synapse formation in CNS neurons lacking all $\alpha_2\delta$ isoforms.

2.4.3.2 β Subunits and Transcriptional Regulation

The first indication of calcium channel independent functions of β subunits came from isolated observations of heterologously expressed β subunits localized in the cell nuclei (Colecraft et al. 2002; Hibino et al. 2003; Beguin et al. 2006).

Interestingly, the truncated chicken β_{4c} isoform associated with heterochromatin protein 1 (HP1) a nuclear protein involved in gene silencing (Hibino et al. 2003). In 2009 we localized the endogenous β_4 isoform in the nuclei of cerebellar granule and Purkinje cells and demonstrated in a skeletal muscle expression system that nuclear targeting of heterologous β subunits is isoform and splice variant specific ($\beta_{4b}>>\beta_{4a}=\beta_3>\beta_{1a}=\beta_{1b}=\beta_{2a}=\beta_{2b}$) and negatively regulated by electrical activity and calcium influx into nerve and muscle cells (Subramanyam et al. 2009). The finding that in immature and quiescent cells β_{4b} accumulated in the nucleus and upon the onset of electrical activity it was released from the nuclei suggested a possible role in activity dependent gene regulation. Very recently Tadmouri et al. reported that β_{4b} associates with the regulatory subunits of protein phosphatases 2A, translocates into the nucleus in an activity dependent manner, where it associates with the tyrosine hydroxilase promoter and histone H3 in complex with HP1 (Tadmouri et al. 2012). Importantly, a truncated β_4 mutant associated with juvenile myoclonic epilepsy failed to complex with B56δ and consequently did not translocate into the nucleus. These findings suggest that the neurological disease phenotype in humans and that of the β_4 knockout mouse are at least in part related to the nuclear function of the β_{4b} subunit, whereas its calcium channel dependent functions may be compensated by other β isoforms. Also β_3 subunits may function in transcriptional regulation. Recently the specific interaction of β_3 with a novel Pax6(S) transcriptional regulator has been described (Zhang et al. 2010). Upon coexpression in Xenopus oocytes β_3 is translocated into the nucleus and suppresses the transcriptional activity of Pax6(S). As Pax6 transcriptional regulators are important during development, a role of this calcium channel independent activity of β in developmental regulation has been suggested. Consistent with function in early development, morpholino knockdown of β_4 in zebrafish embryos blocked epiboly, a reorganization of cells during gastrulation (Ebert et al. 2008). Importantly, this effect could be rescued by coexpression of a β_{4a} isoform with mutated AID binding pocket, again indicative of a calcium channel independent mechanism. So far no direct link of any of these nuclear functions of β subunits to synaptic function has been established. However, because these novel pathways for transcriptional regulation are activity dependent and affect developmental processes, a mechanism by which β subunit signaling provides a feedback loop from overall synaptic activity to synapse efficacy analogous to homeostatic plasticity can be envisioned.

2.4.4 Auxiliary β and $\alpha_2\delta$ Subunits and Neuronal Disease

There is little evidence for an involvement of calcium channel β subunits in neurological disease. Also, with the exception of *lethargic* (β_4-null) mice, mouse mutant and knockout models of β subunits show little to no neurological defects (see box). Loss of function phenotypes can be observed in cell types predominantly

expressing a single β isoform like skeletal (β_1) and cardiac muscle (β_2) or the retina and inner hair cells (β_2). In other cells, including most neurons, expression of other β isoforms seems to compensate the loss of the respective isoform. The β_4 subunit is the notable exception. Mutations resulting in a truncated protein have been linked to juvenile myoclonic epilepsy (Escayg et al. 2000) and the *lethargic* β_4-null mutant mouse develops severe ataxia and epileptic seizures (Burgess et al. 1997). The similarity of this phenotype to that of $Ca_V2.1$ (*tottering, leaner*) (Doyle et al. 1997) and $\alpha_2\delta$-2 (*ducky*) (Barclay et al. 2001) mutants and their predominant expression in cerebellum (see above) indicates that in some cerebellar neurons this set of subunits forms an essential channel complex. Loss of any one of its components cannot be fully compensated by other isoforms. Alternatively, the neurological β_4 phenotype could arise from an exclusive nuclear function of this subunit in gene regulation.

As mentioned above, $\alpha_2\delta$-dependent functions can be exerted as calcium channel subunits on the one hand, and independent of the Ca_V complex on the other. For example meanwhile it is well established that $\alpha_2\delta$-1 is strongly upregulated in dorsal root ganglion neurons in animal models of neuropathic pain (Luo et al. 2001). The beneficial effect of GBP and PG in neuropathic pain (Field et al. 2006) most likely results from impairing excess $\alpha_2\delta$ subunit trafficking (Bauer et al. 2009). As a possible mechanism inhibiting recycling of $\alpha_2\delta$ subunits from the endosomes has been described (Tran-Van-Minh and Dolphin 2010). *straightjacket* mutants also display altered heat nociception and CACNA2D3 ($\alpha_2\delta$-3) single nucleotide polymorphisms (SNPs) in humans have been linked to central pain processing (Neely et al. 2010). This phenotype, which is likely caused by a change in local CNS excitability, could both be explained by a defect in Ca_V trafficking and synapse formation. Mutant (*du, du^{2J}, entla*) and knockout mice for $\alpha_2\delta$-2 display altered morphology and reduced calcium currents in Purkinje cells as well as cerebellar ataxia and absence epilepsy (Barclay et al. 2001; Brill et al. 2004; Ivanov et al. 2004; Donato et al. 2006). In humans the $\alpha_2\delta$-2 gene (CACNA2D2) has been discussed as a potential tumor suppressor gene (Hesson et al. 2007) and in the context of childhood absence epilepsy (Chioza et al. 2009). Indeed, very recently Edvardson et al. identified the first human mutation in the CACNA2D2 gene associated with an early infantile epileptic encephalopathy (Edvardson et al. 2013). A spontaneous mouse mutant of $\alpha_2\delta$-4 (Cacna2d4) causes structural and functional abnormalities of retinal ribbon synapses associated with the loss of rods (Wycisk et al. 2006a) and a human CACNA2D4 mutation underlies a slowly progressing cone dystrophy associated with night blindness (Wycisk et al. 2006b). Finally, clinical applications of GBP and PG provide an important link between $\alpha_2\delta$ subunits and neuronal disease. Besides their effectiveness in neuropathic pain conditions, which is most likely mediated by binding to $\alpha_2\delta$-1 (Field et al. 2006), both drugs have proven efficacy in epilepsy and generalized anxiety disorders (Bryans and Wustrow 1999; Johannessen Landmark 2008). The recently identified interaction of mutant prion protein with $\alpha_2\delta$-1 may provide an essential disease mechanism in the pathophysiology of prion diseases, namely by disrupting cerebellar glutamatergic neurotransmission (Senatore et al. 2012).

Box: Insight into Neuronal Functions of Auxiliary Subunits from Knockout and Mutant Animal Models

- **β_1 (Cacnb1):** Mice with a disruption of the β_1 isoform die after birth due to respiratory failure (Gregg et al. 1996). Similarly the paralyzed zebrafish mutant *relaxed* is a functional β_1 null mutant (Schredelseker et al. 2005) displaying disturbed skeletal muscle function. Rescue of the skeletal muscle phenotype in β_1 knockout mice by expression of the murine β_{1a} cDNA under the control of the human skeletal muscle actin promoter did not show any obvious neuronal phenotype, suggesting that loss of β_1 can be compensated by other β subunits (Ball et al. 2002). Nevertheless, detailed analysis of neuronal phenotypes is still pending.
- **β_2 (Cacnb2):** Mice with a targeted deletion of β_2 die during embryonic development due to cardiac failure (Ball et al. 2002; Weissgerber et al. 2006). Mice in which the lethal phenotype was rescued by the expression of β_2 under a cardiac promoter did not display an obvious CNS phenotype. Nevertheless, these mice displayed altered retinal morphology highlighting the importance of β_2 in membrane expression of $Ca_V1.4$ channels, which are exclusively expressed in the retina. Furthermore these mice are deaf due to reduced $Ca_V1.3$ membrane expression in inner hair cells, revealing their importance in the L-type channel dependent synapse (Neef et al. 2009).
- **β_3 (Cacnb3):** Deletion of the β_3 isoform resulted in reduced N-type currents in the hippocampus, suggesting a preferential interaction of β_3 with $Ca_V2.2$, as well as enhanced LTP and hippocampus-dependent learning. Lower anxiety, increased aggression and nighttime activity further indicate a general imbalance in neuronal calcium handling (Namkung et al. 1998; Murakami et al. 2007; Jeon et al. 2008).
- **β_4 (Cacnb4):** The spontaneous β_4 mouse mutant *lethargic* displays ataxia and epileptic seizures (Burgess et al. 1997) and the major phenotype arises from disrupted P/Q-type signaling in the cerebellum (similar to $Ca_V2.1$ *tottering* and $\alpha_2\delta$-2 *ducky* mutant animals), highlighting the preferential local association of β_4 with $Ca_V2.1$ and $\alpha_2\delta$-2. β_4 mutants display the strongest CNS phenotype of all β knockout mice, suggesting limited potential for compensation by other isoforms. It is further tempting to speculate that this may at least in part relate to the unique property of β_4 subunits in the regulation of gene transcription.
- **$\alpha_2\delta$-1 (Cacna2d1):** As previously inferred from a modeling study (Tuluc et al. 2007), $\alpha_2\delta$-1 knockout mice display reduced cardiac L-type currents (Fuller-Bicer et al. 2009). However, the mice do not show any obvious neuronal phenotype.
- **$\alpha_2\delta$-2 (Cacna2d2):** The naturally occurring $\alpha_2\delta$-2 mutants (*du, du^{2J}, entla*) as well as targeted knockout mice display epilepsy and ataxia

(continued)

(continued)
and show severely impaired cerebellar development (Barclay et al. 2001; Brodbeck et al. 2002; Brill et al. 2004; Ivanov et al. 2004; Donato et al. 2006). Together with the β_4 mutant *lethargic* and the $Ca_V2.1$ mutant *tottering* this mirrors the association of these subunits in the cerebellum. Due to the severe cerebellar phenotype, other CNS functions (e.g., in the hippocampus) have until today not been analyzed.

- **$\alpha_2\delta$-3 (Cacna2d3):** Mice with a targeted deletion of $\alpha_2\delta$-3 display defects in pain processing (Neely et al. 2010), the precise mechanism for this effect has not yet been elucidated. Synapse formation in hippocampal neurons appears to be normal (Nimmervoll et al. submitted). However, these mice have impaired hearing and a reduced auditory startle response, which is likely caused by a defect in synapse formations along the auditory pathway (Pirone et al. 2009). The Drosophila $\alpha_2\delta$-3 mutant *straightjacket* displays defects in presynaptic channel trafficking, motoneuron synapse formation, and altered heat nociception (Dickman et al. 2008; Kurshan et al. 2009; Neely et al. 2010). A mutant of the C. elegans $\alpha_2\delta$ subunit *unc-36* shows impaired synaptic function likely due to impeded presynaptic channel trafficking (Saheki and Bargmann 2009).
- **$\alpha_2\delta$-4 (Cacna2d4):** A spontaneous mouse mutant of $\alpha_2\delta$-4 causes structural and functional abnormalities of retinal ribbon synapses associated with the loss of rods (Wycisk et al. 2006a). The human CACNA2D4 mutation underlies a slowly progressing cone dystrophy associated with night blindness (Wycisk et al. 2006b).

2.5 Conclusion

Moving the focus of calcium channel research from heterologous expression systems to differentiated cells including neurons and to the study of animal models have greatly advanced our understanding of the physiology of auxiliary Ca_V subunits. However, many of the new functional insights have also revealed our limited ability to associate their specific functions to particular molecular entities. This deficit has been further exacerbated by the growing molecular diversity of calcium channel subunits brought about by posttranscriptional modifications like splicing and RNA editing. Therefore, future research first and foremost needs to uncover how specific Ca_V complexes are established in neurons expressing many different isoforms. As outlined above, this will require the detailed study of their expression patterns, their targeting mechanisms and their protein-protein interactions. To uncover these aspects in the context of calcium channel signalling complexes like the presynaptic compartment high- and superresolution microscopy approaches will be necessary. Finally, the static picture of molecular complexes needs to be replaced by one

of highly dynamic signalosomes, in which all the mechanisms mentioned above contribute to an equilibrium of multiple protein-protein interactions that ultimately determines the functional properties of the signalling complex. In the synapse such dynamic calcium channel complexes are critical for the activity-dependent regulation of synaptic strength and ultimately for the ability of our nervous system to learn and store new information.

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