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Review Article

The life cycle of voltage-gated Ca^{2+} channels in neurons: an update on the trafficking of neuronal calcium channels

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Neuronal voltage-gated Ca^{2+} (Ca_V) channels play a critical role in cellular excitability, synaptic transmission, excitation–transcription coupling and activation of intracellular signaling pathways. Ca_V channels are multiprotein complexes and their functional expression in the plasma membrane involves finely tuned mechanisms, including forward trafficking from the endoplasmic reticulum (ER) to the plasma membrane, endocytosis and recycling. Whether genetic or acquired, alterations and defects in the trafficking of neuronal Ca_V channels can have severe physiological consequences. In this review, we address the current evidence concerning the regulatory mechanisms which underlie precise control of neuronal Ca_V channel trafficking and we discuss their potential as therapeutic targets.

Introduction

Calcium (Ca^{2+}) channels mediate numerous important physiological processes, and are abundant in many types of cells [1,2]. In neurons, voltage-gated Ca^{2+} (Ca_V) channels are expressed in most plasma membrane compartments and they are involved in regulating cell excitability, gene transcription and synaptic transmission. Ca_V channels are activated by membrane depolarization and they can be classified into two major categories: high-voltage-activated channels (HVAs), consisting of L-type ($Ca_V1.1$, 1.2, 1.3 and 1.4), P/Q-type ($Ca_V2.1$), N-type ($Ca_V2.2$), and R-type ($Ca_V2.3$) channels, and low-voltage-activated channels (LVAs), which encompass the T-type channels ($Ca_V3.1$, $Ca_V3.2$, $Ca_V3.3$) [3,4]. All HVA channels contain multiple subunits which assemble to form a functional channel complex (Figure 1). These subunits include the pore forming $Ca_V\alpha_1$ subunit and auxiliary $\alpha_2\delta$ and β subunits, and in some cases a γ subunit. Conversely, LVA channels only require a $Ca_V\alpha_1$ subunit to be functional.

Pore forming $Ca_V\alpha_1$ subunits exhibit four repeat domains each containing six transmembrane segments (Figure 1). Crystallography and cryo-EM experiments have provided exquisite details of the atomic structure of Ca_V channels and their auxilliary subunits [5–7]. Segments S1–S4 constitute the voltage-sensing domain and segments S5–S6 form the pore and the selectivity filter. The amino (N) and carboxy (C) termini and the cytoplasmic loops that connect the four transmembrane domains are important domains involved in the modulation of the activity of the channels, as well as forming critical protein interaction platforms that regulate the trafficking of Ca_V channels to the plasma membrane.

Auxilliary β subunits are crucial for the regulation of HVA channel activity through modulation of their biophysical properties [8–11] and the control of their membrane trafficking [8,11–13]. There are four different types of β subunits (encoded by four genes) and they are largely cytoplasmic. However, palmitoylation of the β_{2a} subunit takes place post-translationally at its N-terminus and results in the targeting of the subunit to the plasma membrane [14]. All β subunits consist of five distinct structural regions: the N-terminus, the src homology 3 (SH3) domain, the HOOK domain, the GK domain, and the

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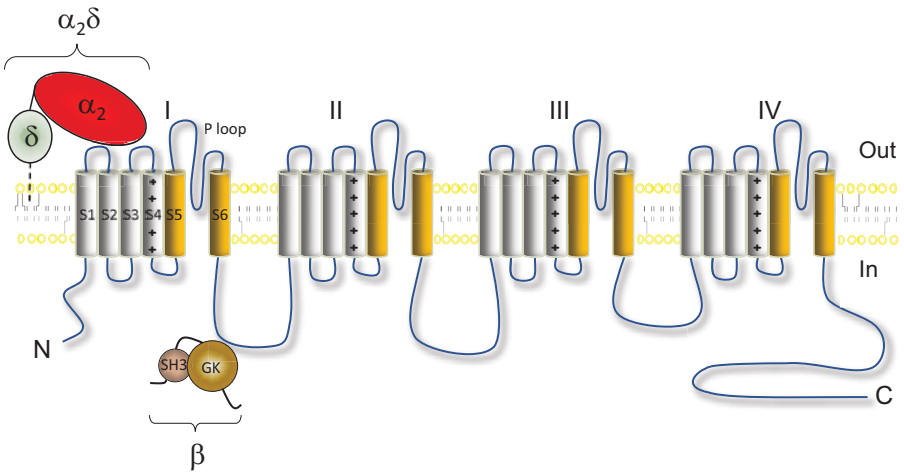

Figure 1. Schematic representation of the structure of Ca_V channels

The $Ca_V\alpha_1$ subunit is formed by four repeat domains (I–IV) each containing six transmembrane segments: S1–S4 constitute the voltage sensor domain (S4 segments contain positively charged residues) and S5–S6 constitute the pore domain (the P loops contain acidic residues that contribute to the selectivity filter of the channel). $Ca_V\alpha_1$ subunits can be associated with auxiliary subunits: an extracellular $\alpha_2\delta$ subunit attached to the plasma membrane by a glycosyl phosphatidylinositol (GPI) anchor and an intracellular β subunit which contain a src homology 3 (SH3) domain and a GK domain.

C-terminus [8,15]. The GK and SH3 domains are highly conserved across the different β subunits, and are connected by a variable HOOK domain. The effects of β subunits on HVA channels are mediated by the GK domain, through a region termed the α Interaction Domain (AID) Binding Pocket (ABP) [16–18]. The ABP binds to a region called the AID domain in the I–II loop of the $Ca_V\alpha_1$ subunit, which contains several key residues that modulate β subunit binding. However, it has also been reported that β subunits most likely interact with other regions of $Ca_V\alpha_1$ subunits [19]. β subunits can bind $Ca_V\alpha_1$ subunits in the endoplasmic reticulum (ER) prior to processing in the Golgi, and the resulting $Ca_V\alpha_1$-β subunit complex is often found to be localized at the plasma membrane [9,20].

Auxilliary $\alpha_2\delta$ subunits are also critical for the trafficking of HVA channels [3,11,21]. There are four different genes ecoding $\alpha_2\delta$ subunits, namely $\alpha_2\delta$-1 to -4 [22]. $\alpha_2\delta$ subunits are extracellular proteins, translated into one precursor that is post-translationaly proteolytically cleaved into α_2 and δ peptides which remain attached by disulfide bonds [23,24]. The δ part of $\alpha_2\delta$ was initially predicted to be a transmembrane protein but it was later demonstrated that δ remains attached to the extracellular leaflet of the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor [7,25–27]. $\alpha_2\delta$ subunits contain different functional domains: a von Willebrand factor A (VWA) domain with a metal ion-dependent adhesion site (MIDAS) and multiple Cache domains [7,28]. Several regions of $\alpha_2\delta$ have been predicted to interact with Ca_V channels based on a cryo-EM study [7] and some of these putative interactions have recently been validated by functional studies [29,30].

Ca_V channel subunits are synthesized by ER-bound ribosomes and inserted into the ER membrane while being synthesized. From the ER membrane, proteins are then trafficked to the plasma membrane via the Golgi network and trafficking endosomes (Figure 2). During their journey to the plasma membrane, $Ca_V\alpha_1$ subunits undergo maturation steps and quality control checks, including association with auxiliary subunits β and $\alpha_2\delta$, that affect their ability to reach the plasma membrane and fulfill their physiological roles. Once at the plasma membrane, the fate of Ca_V channels is determined by the dynamic interactions with anchoring proteins, binding partners and the activity of the neurons. Ca_V channels can then be internalized and either recycled or degraded (Figures 3 and 4). In this review, we will highlight our current knowledge about the trafficking of neuronal Ca_V channels with a focus on the mechanisms that regulate these processes.

Forward trafficking of Ca_V: from ER to plasma membrane

Glycosylation of the $Ca_V\alpha_1$ subunit

Glycosylation in the ER and the Golgi system contributes to the quality control of protein folding [31–33]. N-linked glycosylation corresponds to the transfer of oligosaccharide chains (glycans) on to asparagine residues of newly synthesized proteins in the ER. The N-glycans interact with lectin chaperones such as calnexin and calreticulin to ensure

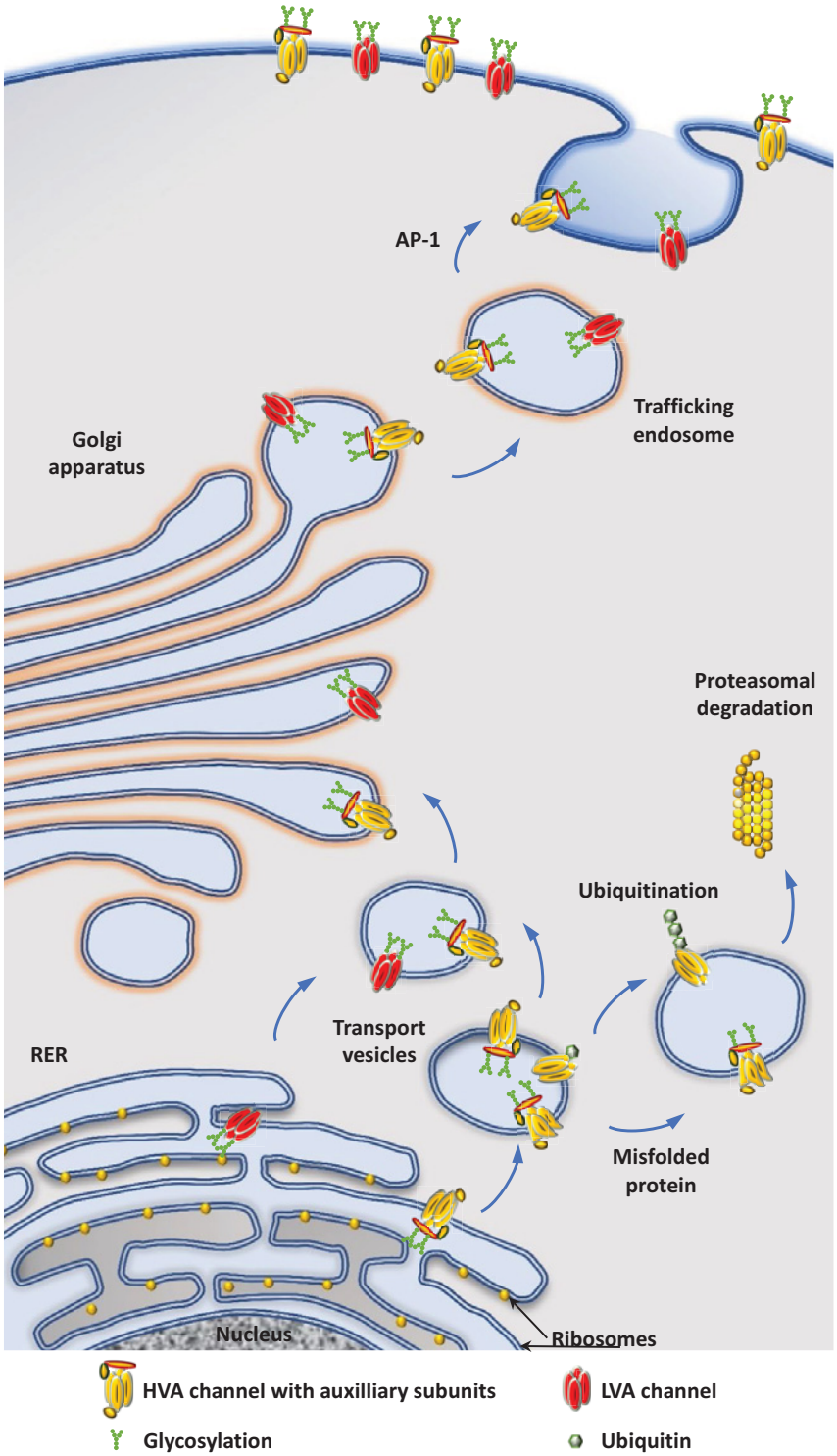

Figure 2. Diagram of forward trafficking mechanisms of Ca_V channels from the ER to the plasma membrane

Newly synthesized peptides are translocated to the rough ER (RER) where they associate with auxiliary subunits and are subjected to post-translational modifications including glycosylation. Ca_V channels are then trafficked to the plasma membrane via the Golgi apparatus and trafficking endosomes. Along the way, misfolded proteins are identified by quality-control mechanisms and targeted for degradation. The association of $Ca_V\alpha_1$ with β subunits prevents the ubiquitination of the $Ca_V\alpha_1$ subunit which protect channels from degradation by the proteasome. The adaptor protein AP1 interacts with $Ca_V\alpha_1$ and contribute to the incorporation of channels to the plasma membrane via clathrin-coated vesicles.

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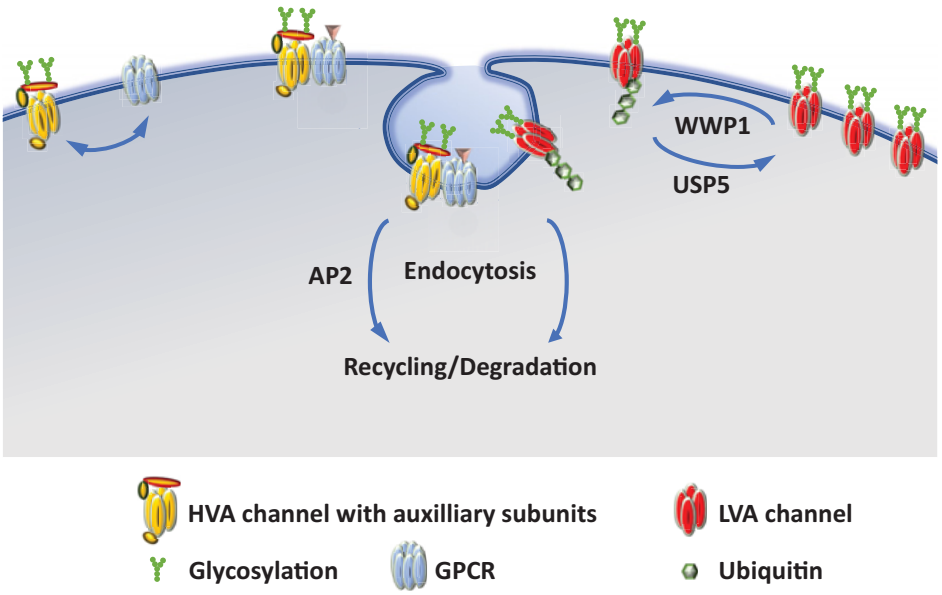

Figure 3. Schematic depiction of the internalization of Ca_V channels

The stability of Ca_V channels at the plasma membrane is determined by the activity of the channel and by the interaction with regulatory proteins. G protein-coupled receptors (GPCRs), like the D2R dopamine receptor, have been shown to directly interact with $Ca_V2.2$ channels and to induce the internalization of the complex when the receptor is activated by its agonist. The adaptor protein 2 (AP2) has been implicated in this internalization process. For $Ca_V3.2$, the balance between ubiquitination/de-ubiquitination is key to the stability of the channels in the plasma membrane. USP5, a de-ubiquitinase, removes ubiquitin from $Ca_V3.2$ increasing the lifetime of the channels at the plasma membrane whereas WWP1, a ubiquitin ligase, transfers ubiquitin to $Ca_V3.2$ and promotes the endocytosis of the ubiquitinated channels. Endocytosed Ca_V channels are then either recycled or degraded.

the selective export of properly folded proteins. Although a critical role of N-glycosylation on the trafficking and function of membrane proteins such as ion channels has first been demonstrated over a decade ago [34–41], it was only recently that its impact on Ca_V channels (mainly $Ca_V3.2$ T-type channels) has attracted more attention [42,43]. Four putative N-glycosylation sites have been identified in extracellular loops of $Ca_V3.2$ channels: N192 in loop 2 of domain I; N271 in loop 3 of domain I; N1466 in loop 3 of domain III; N1710 in loop 2 of domain IV [44,45]. A combination of pharmacological tools and site-directed mutagenesis was used to characterize the role of these glycosylation sites: residues N271 and N1710 are essential for passing quality control as their mutations induce an almost complete loss of protein expression. Although discrepencies have been reported regarding the magnitude of the effect of mutating N1466 and N192 on $Ca_V3.2$ functional expression, it appears that these glycosylation sites are critical for the trafficking of the channels to the plasma membrane and that they also affect the biophysical properties of the channels [44–46]. A recent study has investigated the impact of the double mutation N192Q and N1466Q on the trafficking of $Ca_V3.2$ by scrutinizing lateral mobility (investigated by fluorescence recovery after photobleaching) and internalization (investigated by antibody internalization assay) [47]. These data revealed that whereas lateral mobility is not affected, the internalization rate of $Ca_V3.2$ channels is increased when the glycosylation sites are mutated indicating that the stability of channels at the plasma membrane is reduced [47]. However, further investigations focusing on the net forward trafficking of $Ca_V3.2$ will be needed to ascertain a role of N-glycosylation of $Ca_V3.2$ on its recycling or forward trafficking. As $Ca_V3.2$ channel up-regulation is a common feature in the development and maintenance of multiple pain processes [48], and alterations of $Ca_V3.2$ channels glycosylation have been associated with the development of pain related to diabetes [43,45,49], understanding $Ca_V3.2$ trafficking and the impact of its glycosylation are of significant therapeutic relevance [50].

Auxiliary $\alpha_2\delta$ subunits

The $\alpha_2\delta$ subunits associate with $Ca_V\alpha_1$ subunits in the ER and promote their trafficking to the plasma membrane [3,4]. However, the exact mechanism by which $\alpha_2\delta$ increases the density of Ca_V channels at the plasma membrane is

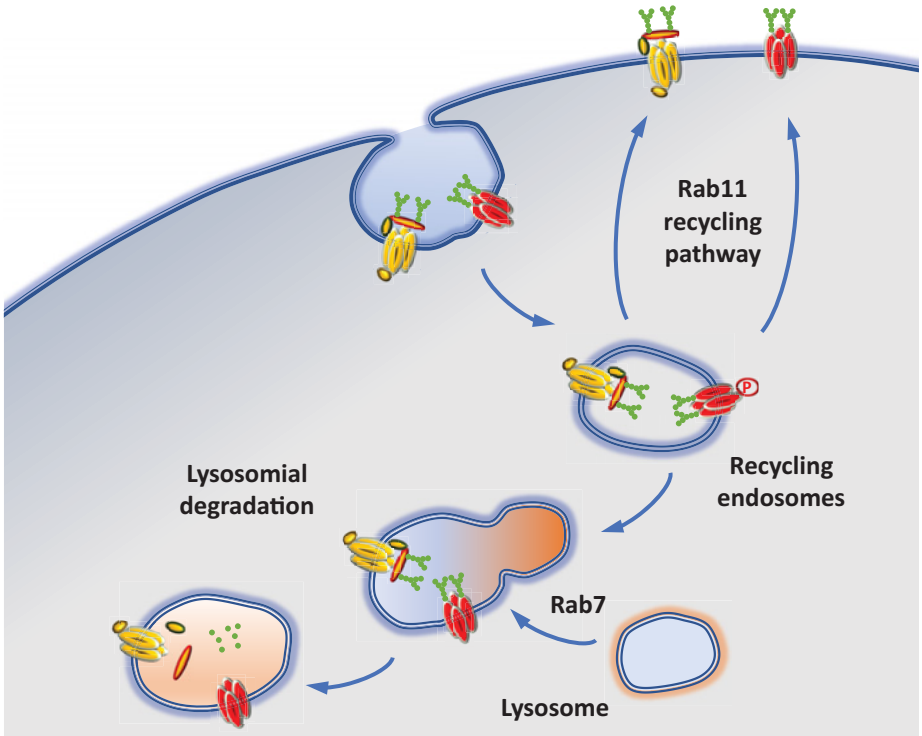

Figure 4. Schematic of the recycling and degradation of Ca_V channels

Endocytosed Ca_V channels are either recycled or degraded. Rab11, a small GTPase that controls key events of vesicular transport, is suspected to be a major player in the recycling of Ca_V to the plasma membrane by interacting either with the $Ca_V\alpha_1$ subunit or with the $\alpha_2\delta$ auxiliary subunit. Following their endocytosis Ca_V channels have been shown to be co-localized with Rab7, a marker for late endosomes and lyzosomes.

still under investigation. Evidence obtained from a neuronal cell line (N2a cells) transiently expressing $Ca_V2.2$ indicated that $\alpha_2\delta$-1 does not affect the endocytosis of the channel [51]. Instead, $\alpha_2\delta$ subunits are suspected to control the trafficking of Ca_V channels either by promoting their transfer from the ER to the plasma membrane or by increasing their recycling.

The $\alpha_2\delta$ subunits are also synthesized by ER-bound ribosomes and translocated in the ER lumen. $\alpha_2\delta$ subunits are highly glycosylated [26,52] and this process is critical for the trafficking of Ca_V channels. It then appears obvious that, as the glycosylation state of $\alpha_2\delta$ subunits affects their trafficking to the plasma membrane, it can consequently affect the trafficking of the pore forming unit [53,54]. Tetreault and colleagues performed an extensive site-directed mutagenesis study of the 16 putative N-glycosylation sites of $\alpha_2\delta$-1 and showed that, in addition to playing a role in stability/quality control and trafficking of $\alpha_2\delta$-1, specific glycosylation sites of $\alpha_2\delta$-1 are involved in the modulation of $Ca_V1.2$ biophysical properties [53].

The $\alpha_2\delta$-1 subunit was shown to interact with the low-density lipoprotein receptor-related protein-1 (LRP1) [55]. When LRP1 is expressed with its chaperone protein, the receptor-associated protein (RAP), it promotes $\alpha_2\delta$-1 glycosylation maturation, trafficking, and cell surface expression. This LRP1/RAP complex also promotes the functional expression of $Ca_V2.2$ (cell surface expression and current density).

Besides glycosylation, $\alpha_2\delta$ is subject to additional post-translational modifications such as the formation of disulfide bonds and the proteolytic cleavage [56]. Disulfide bonds allow α_2 to stay linked to δ and thus to the membrane after the proteolytic cleavage. The proteolytic cleavage of $\alpha_2\delta$ does not appear to affect the trafficking of Ca_V2 channels but plays a role in the fully functional channel complex [57,58].

Auxiliary β subunits

It is well established that β subunits have a direct role in trafficking HVA ($Ca_V1.X$ and $Ca_V2.X$) channels to the plasma membrane. However, the mechanism of how this occurs is yet to be fully elucidated. Initially, it was reported in *Xenopus laevis* oocytes that β subunits co-expressed with $Ca_V2.1$ channels resulted in an increase in Ca^{2+} current

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amplitude [9]. It was hypothesized that β subunit binding to $Ca_V2.1$ α_1 resulted in the masking of an ER retention motif present on the I–II loop [12,59]. However, studies performed on $Ca_V1.2$ and $Ca_V2.2$ did not provide evidence that such an ER retention signal exists in their I–II loop. Indeed, CD4 proteins fused to the I–II linker of $Ca_V1.2$ or $Ca_V2.2$ are efficiently trafficked to the plasma membrane in the absence of β subunits [60]. Furthermore, chimeric channels formed by swapping the I–II linkers from $Ca_V1.2$ or $Ca_V2.2$ to $Ca_V3.1$ α_1 subunits, which do not require β subunits for their plasma membrane targeting, generated larger currents than wild type $Ca_V3.1$ [61,62]. Altogether, these studies support the existence of an ER export signal in the I–II loop of $Ca_V1.2$ and $Ca_V2.2$. Finally, extensive analysis of $Ca_V1.2$ intracellular domains identified ER retention signals in all the other intracellular linkers and in the N- and C-termini [13,60,61]. Current thinking is that when a β subunit binds the the AID of a $Ca_V\alpha_1$ subunit in the ER, conformational changes mask the retention signals and expose the export signal. This then allows the channel complex to be trafficked to the plasma membrane [8,15]. However, questions remain about the function of the I–II linker (ER retention or export signal) between $Ca_V2.1$ and the other HVA channels [59–61]. For example, does this difference point to a $Ca_V2.1$ channel specificity? Further investigation will be needed to confirm this speculation.

β subunits increase the trafficking of Ca_V channels by playing the role of a trafficking switch but it was also shown that they can prevent the degradation of Ca_V channels by the proteasome [60,63]. In heterologous expression systems, β subunits reduce $Ca_V1.2$ degradation by binding to the AID domain and inhibiting its ubiquitination by the E3 ubiquitin ligase RFP2 [60]. In the absence of β subunits, ubiquitinated $Ca_V1.2$ channels interact with the ER-Associated Degradation (ERAD) complex derlin-1/p97 proteins to be targeted to the proteasome for degradation. The role of RFP2, and hence ubiquitination, in controlling $Ca_V1.2$ trafficking to the plasma membrane was confirmed in hippocampal neurons [60]. Similarly, for $Ca_V2.2$ channels it was shown in rat superior cervical ganglia (SCG) neurons that a mutation in the AID domain that prevents the binding of β subunits [64] induced an increase in the channel degradation compared with wildtype $Ca_V2.2$. This effect was blocked by proteasomal inhibitors [63]. It was later shown that the interaction with β subunits prevents the poly-ubiquitination of the $Ca_V2.2$ I–II loop and its proteasomal degradation, thus increasing the forward trafficking of the channel [65].

Nedd4-1, a ubiquitin ligase, was reported to decrease the plasma membrane density of $Ca_V1.2$ in a β-dependent manner through lysosomal degradation [66]. However, the mechanism of action of Nedd4-1, which does not involve a direct ubiquitination of the channel complex, remains to be elucidated.

Furthermore, the phosphorylation state of β subunits can affect Ca_V channel trafficking to the plasma membrane. In COS7 cells and rat dorsal root ganglion (DRG) neurons, Akt, a kinase in the PI3Kγ pathway, was reported to phosphorylate β subunits through a PIP3-dependent mechanism [67]. Akt specifically phosphorylates a serine residue in the C-terminus of β2a leading to an increase in trafficking of the channels ($Ca_V1.2$ and $Ca_V2.2$) to the plasma membrane and an increase in calcium current density [67]. The effect of Akt on $Ca_V1.2$ was later shown to occur also in cardiomyocytes [68]. Altogether, these studies suggest that the phosphorylation of β2a promotes its chaperone role on Ca_V channels.

As we will discuss in the Endocytosis section of this review, G protein-coupled receptors (GPCRs) are potent modulators of Ca_V channel trafficking to the plasma membrane through direct interaction with the $Ca_V\alpha_1$ pore-forming subunit. However, the Growth Hormone Secretagogue Receptor type 1a (GHSR), a GPCR that constitutively controls Ca_V current density via $G_{i/0}$ activation, was recently shown to exert its effect by promoting the retention of Ca_V channels in the ER [69,70]. Intriguingly, this effect of GHSR on Ca_V channels depends on the presence of β subunits but does not rely on the interaction of β with the AID of Ca_V channels. Further studies will be needed to identify the molecular mechanism at play in this signaling pathway.

Adaptor protein 1

From the surface of the trans-Golgi network, clathrin-coated vesicles are formed by the recruitment of clathrin via heterotetrameric Adaptor Protein 1 (AP1) complexes [71]. Clathrin-coated vesicles are responsible for the transport of cargo molecules to the plasma membrane. Membrane-bound AP1 complexes interact with sorting signals (YxxΦ and [DE]xxxL[LI], where x is any amino acid and Φ is a bulky hydrophobic residue) contained within the cytosolic tails of transmembrane proteins. Such sorting signals have been identified in the proximal C-terminus of $Ca_V2.2$ [72]. The mutation of these consensus motifs in $Ca_V2.2$, the knockdown of one component of the AP1 complex (AP1 γ) using shRNA, and the expression of a dominant negative form of one component of AP1 complex (AP1 σ) all reduced the cell surface expression of $Ca_V2.2$ in N2a cells and in DRG neurons. These findings demonstrate the functional involvement of the AP1 complex in the trafficking of $Ca_V2.2$ channels to the plasma membrane [72]. AP1 binding motifs are located in exon 37 of $Ca_V2.2$. Exon 37 is subject to alternative splicing and can generate 2 mutually exclusive variants (37a and 37b) [73,74]: exon 37a contains two AP1 consensus sites whereas exon 37b contains only

one noncanonical AP1 site [72]. It is worth noting that cell surface expression of $Ca_V2.2$ channels containing exon 37a is higher than $Ca_V2.2$ channels containing exon 37b which reinforces the importance of this region for the trafficking of $Ca_V2.2$ to the plasma membrane. Moreover, exon 37a is selectively expressed in peripheral nociceptive neurons and its expression is critical for pain signaling [73,75]. AP1 consensus binding motifs can also be found in the proximal C-terminus of $Ca_V1.3$, $Ca_V1.4$ and $Ca_V2.1$ (exon37a) which suggests that forward trafficking of these channels may also be AP1 dependent. Altogether, these data highlight the possibility that targeting AP1/$Ca_V2.2$ interactions may serve as a therapeutic approach towards pain modulation.

Fragile X mental retardation protein

The Fragile X mental retardation protein (FMRP) was shown to control the functional expression of ion channels [76–78]. FMRP affects $Ca_V2.2$ channels in neurons by directly interacting with intracellular domains of $Ca_V2.2$, including its C-terminus [79]. In a recent study (using $Ca_V2.2$ channels with a tandem α-bungarotoxin binding site (BBS) tag in an extracellular loop expressed in N2a cells), FMRP was shown to reduce the trafficking of the channels between the Golgi network and the plasma membrane [80]. Although the exact binding domain of FMRP on the C-terminus of $Ca_V2.2$ still has to be identified, it is possible that FMRP interferes with the binding of the AP1 complex to the $Ca_V2.2$ C-terminus, thereby affecting its forward trafficking as a consequence.

Stac proteins

The Stac3 (SH3- and cysteine-rich domains) protein is essential for EC coupling in skeletal muscle [81,82]. The functional interaction between Stac3 and $Ca_V1.1$ induces an increase in channel density in the plasma membrane and alters the kinetics of the $Ca_V1.1$-generated current in tsA-201 cells [83]. Stac proteins were also shown to alter the Ca^{2+}-dependent inactivation of neuronal L-type channels $Ca_V1.2$ and $Ca_V1.3$, however Stac proteins have no effect on the trafficking of these channels [84,85]. Finally, whereas no effect were reported on non L-type channels ($Ca_V2.1$), Stac1 was shown to increase the expression of $Ca_V3.2$ [86]. Further studies will be needed to determine whether Stac proteins increase the forward trafficking or the stability of these channels at the plasma membrane.

Truncated channels and mutation of the Ca^{2+}-binding site in the pore

Genes encoding $Ca_V\alpha_1$ subunits are transcribed into pre-messenger RNA that is subject to cell specific and developmentally regulated alternative splicing [73,87–92]. Splicing of $Ca_V\alpha_1$ subunits has the ability to generate a multitude of full-length fully functional channels. However, alternative splicing can also give rise to truncated proteins with altered or no channel activity. Functional studies performed on $Ca_V1.1$, $Ca_V1.2$ and $Ca_V2.1$ have shown that truncated channels have physiological relevance by controlling the expression of full-length Ca_V channels [93–96]. Moreover, mutations that result in truncations of $Ca_V\alpha_1$ subunits are suspected to cause pathological states. For example, in episodic ataxia type-2 (EA-2), an autosomal dominant disorder, mutations in the gene *CACNA1A* that encodes $Ca_V2.1$ predict truncated forms of this channel [97,98]. The expression of a truncated channel, either physiological or pathological, was shown to have a dominant-negative effect on co-expressed full-length channels [95,98]. Indeed, it has been shown that truncated $Ca_V2.2$ and $Ca_V2.1$ subunits interact with the full-length channels in the ER. The complex is then either recognized as misfolded proteins which activates a component of the unfolded protein response (UPR) inducing translational arrest [99,100] or targeting for degradation by the proteasome [101]. The N-terminus of the channel is key for the interaction between truncated and wildtype channels and disrupting this interaction has been considered as a potential therapeutic intervention [102,103].

A recent study has investigated the role of the selectivity filter of $Ca_V2.1$ and $Ca_V2.2$ in the trafficking of the channels to the plasma membrane [104]. This study shows that Ca^{2+}-binding sites in the selectivity filter have to be preserved for the channel to be optimally trafficked to the plasma membrane and the authors hypothesized that Ca^{2+} binding to the pore is required for the proper folding of the channel in the ER and therefore for its trafficking.

Calmodulin

The role of calmodulin (CaM) in the regulation of Ca^{2+}-dependent inactivation of Ca_V has been extensively studied [105]. However, CaM involvment in the trafficking of Ca_V remains unclear. CaM is able to bind several motifs in the C-terminus of $Ca_V1.X$ and $Ca_V2.X$ channels [105] and the deletion of these CaM binding motifs in $Ca_V1.2$ was shown to abolish the cell surface expression of the channels [13] and to alter $Ca_V1.2$ current amplitude [106–108] suggesting that CaM can modulate the trafficking of $Ca_V1.2$ channels to the plasma membrane. However, a more recent study challenged this conclusion [109]. Bourdin and colleagues used tsA-201 cells to express an extracellularly tagged $Ca_V1.2$ and examined the effect of CaM and a dominant negative CaM on its cell surface expression [109].

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They quantified $Ca_V1.2$ plasma membrane expression by using fluorescence-activated cell sorting analysis. They did not observe an effect of CaM on $Ca_V1.2$ cell surface expression, thus concluding that CaM is not essential for the trafficking of $Ca_V1.2$ channels [109]. Overall, it can be argued that deletions and/or mutations in the C-terminus of $Ca_V1.2$ channel can affect its trafficking either by disrupting trafficking signals, for example an ER export signal [60], or by affecting the folding of the protein [104], and caution should be exerciced when interpreting the results of such experiments.

Fully mature channels reach the plasma membrane as a protein complex formed by a main $Ca_V\alpha_1$ subunit and auxiliary subunits, glycosylated and associated with binding partners. These complexes are now able to play their physiological role in letting Ca^{2+} flow inside the cell, modulating the excitability of the neurons and activating signaling pathways. Their lifetime at the plasma membrane is then dictated by the cell's activity, the stability of the interactions with their existing partners and the interactions with new ones.

Endocytosis and recycling of Ca_V

GPCRs

GPCRs play critical roles in modulating the activity of Ca_V channels [4]. GPCRs have been described as part of signaling complexes together with Ca_Vs, including $Ca_V1.2$ and β-2 adrenergic receptors [110,111], $Ca_V2.1$ and mGluR1 [112], $Ca_V2.2$ and opioid receptors, dopamine receptors (D1R and D2R), $GABA_B$ receptors, and MT1 melatonin receptors [113–120]. GPCRs activated by their specific agonist bind to a heterotrimeric G protein. This is followed by the exchange of GDP for GTP and dissociation of the G protein into GαGTP and Gβγ. G protein-mediated regulation of Ca_V channels affects their biophysical properties [4,121–124]. For example, Gα(s)-GTP activated by β-2 adrenergic receptors in neurons triggers a cAMP/PKA cascade which culminates in an increase in L-type currents [110,125]. The Gβγ dimer can also trigger specific downstream events including the modulation of Ca_V channel activity. Indeed, Gβγ has been shown to directly interact with intracellular domains of the $Ca_V2.X$ family [126,127] and $Ca_V3.2$ channels [128,129]. For $Ca_V2.X$ channels, Gβγ interacts with the I–II loop and the N-terminus domain and it induces voltage-dependent inhibition [126,127]. For $Ca_V3.2$ channels, Gβγ interacts with the II–III loop and reduces the open probability of the channel [128,129].

While G protein-mediated effects of GPCRs modulate the biophysical properties of Ca_V channels, receptors themselves, including ORL1, D1R and D2R, have been shown to control the cell surface expression of the channels, and this can occur through both ligand-independent and ligand-dependent effects. For the ligand-independent effect, the co-expression of several types of GPCRs (ORL1, D1R and D2R) has been reported to increase the number of $Ca_V2.2$ channels at the plasma membrane [72,114,116,117]. For D1R and ORL1, the interaction with $Ca_V2.2$ occurs through direct binding of intracellular regions of the receptors with the proximal C-terminus of the channels [113,116]. Although they still have to be experimentally demonstrated, several mechanisms have been proposed to explain the increase in channel plasma membrane expression: the receptors could mask an ER retention signal contained within the C-terminus of $Ca_V2.2$ [60] and/or the receptor itself could confer an additional trafficking motif to the channel complex. Moreover, a D2R-dependent increase in $Ca_V2.2e37b$ cell surface expression in N2a cells has been linked to a reduction in the rate of endocytosis [72]. The molecular mechanism involved in this latter effect has yet to be identified, however, as it only occurs for e37b and not e37a, this suggests the presence of a specific interaction motif with D2R within protein sequence encoded by exon 37b. For the ligand-dependent effect, the activation of the receptor induces the internalization of the receptor/channel complex. This effect was shown for ORL1, D1 and D2 receptors [72,114,116,117]. Interestingly, due to the ability of ORL1 to heterodimerize with opioid receptors [130], activated opioid receptors are also able to co-internalize with $Ca_V2.2$ channels when they are co-expressed with ORL1 [115]. The mechanism of internalization of the complex has not yet been fully elucidated. Nonetheless, for D2R and $Ca_V2.2$, the internalization of the activated complex relies on both the AP2μ2 protein and an AP2 binding motif in the C-terminus of $Ca_V2.2$ which suggests a clathrin-mediated endocytosis via β-arrestin [72].

RGK proteins

RGK GTPases are a family of small GTPases consisting of Rem, Rem2, Rad and Gem/Kir [131–134] and they all have been shown to inhibit $Ca_V1.X$ and $Ca_V2.X$ channels [8,135]. RGK proteins can utilize multiple mechanisms to inhibit $Ca_V1.X$ and $Ca_V2.X$ channels: they can affect the channel's cell surface expression, their open probability and they can immobilize the voltage sensor of the channel [135,136]. The respective contribution of each mechanism to the inhibitory effect of RGK on $Ca_V1.X$ and $Ca_V2.X$ is thought to be dependent on the combination RGK/channel types that a cell expresses [135]. Precisely how RGK proteins affect the trafficking of Ca_V channels is still not fully understood.

The first evidence of an inhibitory effect of RGK proteins on Ca_V channels was presented by Béguin and colleagues [137]. These authors identified Gem as a binding partner for β subunits (β_1, β_2 and β_3) and then showed that the expression of Gem in *Xenopus* oocytes virtually abolished the currents generated by both $Ca_V1.2$ and $Ca_V1.3$ when co-expressed with β_1, β_2 or β_3 subunits. Finally, they correlated the reduction in Ca^{2+} current with a reduction in $Ca_V1.2$ cell surface expression. Indeed, they showed that the co-expression of Gem with $Ca_V1.2$/β-3 in HEK 293 cells prevents $Ca_V1.2$ channels from reaching the plasma membrane and this leads to the formation of intracellular channel aggregates. These results suggested that Gem competed with $Ca_V\alpha_1$ subunits for binding of β subunits and consequently prevented $Ca_V\alpha_1$ subunits from being trafficked to the plasma membrane. However, this hypothesis was challenged by a subsequent study by Yang and Colecraft [136] who investigated the inhibitory mechanism of Rem on $Ca_V1.2$ channels co-expressed with β_{2a}. The authors generated a $Ca_V1.2$ channel tagged with an α-bungarotoxin binding site in an extracellular loop to monitor its cell surface expression in HEK 293 cells. They used a combination of biotinylated α-bungarotoxin and streptavidin coupled to quantum dots to show that the expression of Rem reduced $Ca_V1.2$ cell surface expression by activating a dynamin-dependent mechanism. This suggested that Rem affects $Ca_V1.2$ surface expression by increasing its internalization. They also showed that Rem-dependent internalization relied on the interaction with β_{2a} since the reduction in $Ca_V1.2$ surface expression was not as noticeable in the absence of the β subunit. In subsequent studies, it was shown that RGK proteins can exert both β-binding-dependent and β-binding-independent inhibition of Ca_V channels [138–140]. Indeed, when a mutant β_{2a} subunit lacking the ability to bind Rem is co-expressed with $Ca_V1.2$ and Rem in HEK 293 cells, $Ca_V1.2$ current is still reduced [140]. Conversely, mutant Rem and Rad constructs that do not interact with β subunits are still able to inhibit $Ca_V1.2$ currents [139]. Rem-dependent internalization of $Ca_V1.2$ channels was shown to be due to a β-binding-dependent mechanism [140].

For years the mechanism by which β-adrenergic receptor activation increases whole cell L-type calcium currents has been subject to intense investigation and debate. It was recently uncovered in cardiomyocytes that Rad can be phosphorylated by protein kinase A (PKA). This causes a disruption of the interaction between Rad and β-subunits and relieves Rab-dependent inihibition of $Ca_V1.2$ channels [141], thus giving rise to larger L-type currents. This β-binding-dependent inhibition of Rad affects the gating of $Ca_V1.2$ channels rather than their trafficking to the plasma membrane [141,142]. PKA activation is triggered in cardiomyocytes by a β-adrenergic receptor pathway and plays a crucial role in the fight-or-flight response [143,144]. A similar PKA-dependent phosphorylation effect was demonstrated between Rad (and Rem) and $Ca_V1.3$ and $Ca_V2.2$ channels expressed in HEK 293T cells [141]. It would be of great interest to determine whether similar mechanisms occur in neurons.

The development of genetically encoded Ca_V channel inhibitors has been one of the foci of the Colecraft lab for many years [145]. Understanding the mechanisms by which RGK proteins inhibit Ca_V channels has allowed them to engineer RGK proteins that specifically inhibit subtypes of Ca_V channels in cardiomyocytes and in neurons, i.e. $Ca_V1.2$ and $Ca_V2.2$ [139]. In cardiomyocytes, it was shown that by targeting Rem expression to caveolae, only Ca_V channels localized to caveolae were inhibited, leaving $Ca_V1.2$ channels responsible for excitation–contraction coupling in the T-tubule virtually unaffected [146]. Would a similar subcellular targeting strategy of RGK proteins be a means for inhibiting specific neuronal subtypes of Ca_V? This could provide a powerful tool to tune synaptic transmission by targeting presynaptic $Ca_V2.2$ channels without affecting somatic $Ca_V1.2$ channels.

Other interactors affecting Ca_V channel endocytosis

While the forward trafficking effects of β subunits have been under continuous inquiry, whether β subunits have a role in endocytosis of $Ca_V1.2$ channels has yet to be thoroughly explored. A study by Hidalgo and colleagues first showed that the SH3 domain of the β subunit can increase the internalization of $Ca_V1.2$ in *Xenopus* oocytes through a dynamin-dependent interaction [147]. They later found that homodimerization of the β-SH3 domain was necessary for $Ca_V1.2$ endocytosis [148]. The endocytosis of $Ca_V1.2$ occurs through the channel binding to a polyproline motif on the dynamin. Thus, there is evidence that the SH3 domain of β subunits has a role in modulating the endocytosis of Ca_V, however further research is required to determine the net impact on $Ca_V1.2$ surface expression when considering the forward trafficking effect of full-lenght β subunits.

In hippocampal neurons, α-actinin, which binds to F-actin, was shown to stabilize $Ca_V1.2$ channels at the plasma membrane by preventing their endocytosis [149]. In resting conditions, α-actinin and apo-CaM (Ca^{2+}-free CaM) both bind to site in the C-terminus domain of $Ca_V1.2$ (IQ CaM binding domain) [144,149,150]. During prolonged activity, the influx of Ca^{2+} increases the affinity of CaM for the C-terminus $Ca_V1.2$ and displaces the binding of α-actinin, thereby initiating the endocytosis of $Ca_V1.2$. Interestingly, the tumor suppressor eIF3e was shown to be responsible for a Ca^{2+}-induced internalization of $Ca_V1.2$ [151]. It was then suggested that the displacement of α-actinin

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from the $Ca_V1.2$ C-terminus could induce conformational changes that would allow eIF3e to bind to the intracellular II–III loop of $Ca_V1.2$ and then trigger its endocytosis [149]. It is also worth noting the presence of a putative AP2 binding site upstream of the IQ motif in $Ca_V1.2$ C-terminus that can be unmasked when Ca^{2+} binds to apo-CaM [72].

The stromal interaction molecule 1 (STIM-1), the main activator of store-operated Ca^{2+} channels, was shown to directly interact with the C-terminus of $Ca_V1.2$ and reduce its plasma membrane density [152,153]. In hippocampal neurons, STIM-1 affects the depolarization-induced opening of $Ca_V1.2$ by both acutely inhibiting its gating and increasing its endocytosis via a dynamin-dependent mechanism [153]. The interaction STIM-1/$Ca_V1.2$ was recently investigated in the context of synaptic plasticity in dendritic spines of hippocampal neurons [154]. In this study, the authors showed that the depolarization induced by a brief application of glutamate (15 s) triggers a STIM-1 dependent inhibition of L-type current amplitude. However, this inhibition of L-type current did not involve internalization of the channel as blockers of endocytosis, such as Dyngo4a and Pitstop, did not prevent the reduction in L-type current amplitude. Altogether, these studies suggest that STIM-1 can control $Ca_V1.2$ channel activity by different mechanisms depending on the intensity of the stimulus: for brief stimulation, STIM-1 reduces $Ca_V1.2$ activity, and for sustained stimuli, STIM-1 induces the internalization of the channels. Thus, STIM-1 provides an important negative feedback mechanism for Ca^{2+} influx.

As noted above, $Ca_V1.2$ and $Ca_V2.2$ channel surface expression is modulated by ubiquitination, leading to proteasomal degradation. $Ca_V3.2$ surface expression is also dependent on ubiquitination, with de-ubiquitinated channels being more stable at the plasma membrane [155]. Two ubiquitin ligases, WWP1 and WWP2, expressed at the cell surface and USP5, a de-ubiquitinase, are critical for the balance ubiquitination/de-ubiquitination of two motifs in the intracellular III–IV linker of $Ca_V3.2$. Interestingly, USP5 is up-regulated in animal models of chronic pain and this up-regulation has been linked to the increase in $Ca_V3.2$ channel activity and its pro-nociceptive effect [155]. The exact mechanism of how the balance ubiquitination/de-ubiquitination of $Ca_V3.2$ channels affect their surface expression is still to be unravelled. However, based on how Nedd4, an E3 ligase that belongs to the same family as WWP1 and WWP2 regulates the epithelial sodium channel ENaC [156], it is likely that the regulation of $Ca_V3.2$ involves an endocytic mechanism. It is worth noting that the ubiquitination state of $Ca_V3.2$ is modulated by the reversible post-translational addition of small ubiquitin-related modifier (SUMO) peptide on USP5 [157]. Indeed, it has been shown that USP5 SUMOylation decreases $Ca_V3.2$/USP5 interaction affinity and then favors $Ca_V3.2$ ubiquitination and its degradation.

The collapsin response mediator protein 2 (CRMP2) has been shown to interact with the intracellular I–II loop and C-terminus of $Ca_V2.2$ and to increase its cell surface expression [158,159]. The mechanism of action of CRMP2 on $Ca_V2.2$ has not yet been fully identified, but it may prevent $Ca_V2.2$ endocytosis as it does with $Na_v1.7$ channels [160–163]. The effect of CRMP2 on $Ca_V2.2$ plasma membrane stability is modulated by post-translational modifications of CRMP2 like phosphorylation and SUMOylation [164,165].

The Ca^{2+} channel and chemotaxis receptor (cache) domain containing 1 protein (Cachd1), was shown to increase $Ca_V2.2$ cell surface expression in N2a cells and in hippocampal neurons [29]. Cachd1 protein was identified as an α_2-δ like protein based on its structural homologies with α_2-δs: it contains two Cache domains and a VWA domain although with a non-conserved MIDAS motif [166,167]. As opposed to α_2-δ proteins which affect the forward trafficking of the channel, Cachd1 modifies $Ca_V2.2$ trafficking by reducing the rate of endocytosis of the channels [29]. Cachd1 protein was also shown to increase $Ca_V3.1$ surface expression in HEK cells and to induce a T-type mediated increase in cell excitability in hippocampal neurons [168]. However, the mechanism by which Cachd1 modulates the trafficking of $Ca_V3.X$ channels was not investigated [168].

Functional and proteomic analyses of neuronal membranes have revealed a close proximity between $Ca_V2.X$ channels and voltage- and Ca^{2+}-activated potassium (BK) channels [169,170]. More recently, it was demonstrated that BK channels could directly interact with the auxiliary $\alpha_2\delta$-1 subunit and reduce $Ca_V2.2$ plasma membrane trafficking [30]. The co-expression of BK channels with $Ca_V2.2$/$\alpha_2\delta$-1 induces the accumulation of $Ca_V2.2$ channels in Rab7-positive intracellular vesicles, a marker for late endosomes and lysosomes, suggesting that BK channels increase the internalization of $Ca_V2.2$ channels [30]. These results also suggest that the interaction with BK channels occurs only when the $Ca_V2.2$/$\alpha_2\delta$-1 complex has reached the plasma membrane. Furthermore, the fact that BK channels outcompete $Ca_V2.2$ for the binding of $\alpha_2\delta$-1 and increase $Ca_V2.2$ endocytosis is in favor of the idea that $\alpha_2\delta$-1 has to remain associated with $Ca_V2.2$ for the channel complex to stay stably expressed at the plasma membrane [51].

Once they have been endocytosed, channels are targeted either for recycling or for degradation (Figure 4). Very few studies have focused on the pathways involved in the recycling of Ca_Vs. The auxiliary $\alpha_2\delta$-2 subunit has been shown to be recycled by a Rab11-dependent pathway which controls $Ca_V2.1$ current density in tsA-201 cells [171]. In the cardiomyocyte cell line HL-1, $Ca_V1.2$ plasma membrane expression was shown to be dependent on the recycling

of the channel via a Rab11a-dependent pathway [172]. However, in arterial smooth muscle cells, a Rab25-dependent pathway was shown to be involved in the recycling of $Ca_V1.2$ channels [173]. Altogether, these studies suggest a cell type-specific mechanism and further investigation will be needed to identify the pathways involved in the recycling of $Ca_V1.X$ channels in neurons.

The neuronal actin-binding protein Kelch-like 1 has been identified as a regulator of T-type channel expression [174–177]. In heterologous expression systems, Kelch-like 1 was shown to increase $Ca_V3.2$ cell surface targeting in an actin F-dependent manner [177]. The Kelch-like 1 effect on T-type channels was prevented by the co-expression of the dominant negative Rab11-S25N, suggesting the involvement of a Rab11-dependent recycling endosomal pathway [177]. Interestingly, a Rab11-dependent pathway also appears to be involved in the up-regulation of $Ca_V3.2$ channel expression by homocysteine [178]. This latter effect on the recycling of $Ca_V3.2$ channels relies on the phosphorylation of serine residues located in intracellular domains (loop I–II, loop II–III and C-terminus) by protein kinase C [178]. It is noteworthy that although protein phosphorylation affects various aspects of Ca_V channel function [4], very few studies have reported effects on the trafficking of the channels.

Conclusion

The trafficking of Ca_V channels is tightly regulated such that channels can be expressed where and when they are physiologically relevant. In this review we focused on mechanisms that control the trafficking of neuronal Ca_V channels from the ER to the plasma membrane, their stability at the plasma membrane and their recycling to intracellular compartments (Figures 2-4). While our understanding of the life cycle of Ca_V channels has greatly improved, gaps still remain. For example, how Ca_V channels are targeted to the trafficking endosomes and conveyed to specific neuronal subcellular locations is still not fully understood. Moreover, Rab11 has been involved in $\alpha_2\delta$-2 recycling [171] but we have no experimental evidence whether neuronal $Ca_V\alpha_1$ subunits are taken up by the same pathway or one of the many other existing recycling pathways [179,180]. This is a crucial issue as defects in Ca_V trafficking have been linked to pathological conditions such as neuropathic pain and ataxia, and deciphering the intricate mechanisms of Ca_V trafficking could allow the development of strategies to correct these defects.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Abbreviations

ABP, α interaction domain binding pocket; AID, α interaction domain; AP1, Adaptor protein 1; Cachd1, Ca^{2+} channel and chemotaxis receptor domain containing 1 protein; cache, Ca^{2+} channel and chemotaxis receptor; CaM, calmodulin; Ca_V, voltage-gated Ca^{2+}; CRMP2, collapsin response mediator protein 2; cryo-EM, cryogenic electron microscopy; DRG, dorsal root ganglion; eiF3E, Eukaryotic translation initiation factor 3 subunit E; ER, endoplasmic reticulum; FMRP, Fragile X mental retardation protein; GHSR, growth hormone secretagogue receptor type 1a; GPCR, G protein-coupled receptor; HVA, high-voltage-activated channel; LRP1, lipoprotein receptor-related protein-1; LVA, low-voltage-activated channel; MIDAS, metal ion-dependent adhesion site; PIP3, Phosphatidylinositol (3,4,5)-trisphosphate; PKA, protein kinase A; RAP, receptor-associated protein; SH3, src homology 3; Stac3, SH3- and cysteine-rich domain; STIM-1, stromal interaction molecule 1; SUMO, small ubiquitin-related modifier; VWA, von Willebrand factor A.

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