Review Article

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

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Neuronal voltage-gated Ca\textsuperscript{2+} (CaV) channels play a critical role in cellular excitability, synaptic transmission, excitation–transcription coupling and activation of intracellular signaling pathways. CaV 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 CaV channels can have severe physiological consequences. In this review, we address the current evidence concerning the regulatory mechanisms which underlie precise control of neuronal CaV channel trafficking and we discuss their potential as therapeutic targets.

Introduction

Calcium (Ca\textsuperscript{2+}) channels mediate numerous important physiological processes, and are abundant in many types of cells [1,2]. In neurons, voltage-gated Ca\textsuperscript{2+} (CaV) channels are expressed in most plasma membrane compartments and they are involved in regulating cell excitability, gene transcription and synaptic transmission. CaV channels are activated by membrane depolarization and they can be classified into two major categories: high-voltage-activated channels (HVAs), consisting of L-type (CaV\textsubscript{1.1}, 1.2, 1.3 and 1.4), P/Q-type (CaV\textsubscript{2.1}), N-type (CaV\textsubscript{2.2}), and R-type (CaV\textsubscript{2.3}) channels, and low-voltage-activated channels (LVAs), which encompass the T-type channels (CaV\textsubscript{3.1}, CaV\textsubscript{3.2}, CaV\textsubscript{3.3}) [3,4]. All HVA channels contain multiple subunits which assemble to form a functional channel complex (Figure 1). These subunits include the pore forming CaV\textsubscript{\alpha\textsubscript{1}} subunit and auxiliary \alpha\textsubscript{2}\delta and \beta subunits, and in some cases a \gamma subunit. Conversely, LVA channels only require a CaV\textsubscript{\alpha\textsubscript{1}} subunit to be functional.

Pore forming CaV\textsubscript{\alpha\textsubscript{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 CaV channels and their auxiliary 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 CaV channels to the plasma membrane.

Auxiliary \beta 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 \beta subunits (encoded by four genes) and they are largely cytoplasmic. However, palmitoylation of the \beta\textsubscript{2a} subunit takes place post-translationally at its N-terminus and results in the targeting of the subunit to the plasma membrane [14]. All \beta subunits consist of five distinct structural regions: the N-terminus, the src homology 3 (SH3) domain, the HOOK domain, the GK domain, and the
Figure 1. Schematic representation of the structure of CaV channels
The CaVα1 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). CaVα1 subunits can be associated with auxiliary subunits: an extracellular αδ 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 CaVα1 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 CaVα1 subunits [19]. β subunits can bind CaVα1 subunits in the endoplasmic reticulum (ER) prior to processing in the Golgi, and the resulting CaVα1–β subunit complex is often found to be localized at the plasma membrane [9,20].

Auxiliary αδ subunits are also critical for the trafficking of HVA channels [3,11,21]. There are four different genes encoding αδ subunits, namely αδ-1 to -4 [22]. αδ subunits are extracellular proteins, translated into one precursor that is post-translationally proteolytically cleaved into α2 and δ peptides which remain attached by disulfide bonds [23,24]. The δ part of αδ 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]. αδ 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 αδ have been predicted to interact with CaV channels based on a cryo-EM study [7] and some of these putative interactions have recently been validated by functional studies [29,30].

CaV 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, CaVα1 subunits undergo maturation steps and quality control checks, including association with auxiliary subunits β and αδ, that affect their ability to reach the plasma membrane and fulfill their physiological roles. Once at the plasma membrane, the fate of CaV channels is determined by the dynamic interactions with anchoring proteins, binding partners and the activity of the neurons. CaV 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 CaV channels with a focus on the mechanisms that regulate these processes.

Forward trafficking of CaV: from ER to plasma membrane
Glycosylation of the CaVα1 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 CaV 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. CaV 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 CaV$_{\alpha 1}$ with $\beta$ subunits prevents the ubiquitination of the CaV$_{\alpha 1}$ subunit which protect channels from degradation by the proteasome. The adaptor protein AP1 interacts with CaV$_{\alpha 1}$ 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 CaV channels

The stability of CaV 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 CaV2.2 channels and to induce the internalization of the complex when the receptor is activated by its agonist. The adaptor protein2 (AP2) has been implicated in this internalization process. For CaV3.2, 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 CaV3.2 increasing the lifetime of the channels at the plasma membrane whereas WWP1, a ubiquitin ligase, transfers ubiquitin to CaV3.2 and promotes the endocytosis of the ubiquitinated channels. Endocytosed CaV channels are then either recycled or degraded.

Auxiliary α2δ subunits

The α2δ subunits associate with CaV α1 subunits in the ER and promote their trafficking to the plasma membrane [3,4]. However, the exact mechanism by which α2δ increases the density of CaV channels at the plasma membrane is
Figure 4. Schematic of the recycling and degradation of CaV channels
Endocytosed CaV 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 CaV to the plasma membrane by interacting either with the CaVα1 subunit or with the α2δ auxiliary subunit. Following their endocytosis CaV channels have been shown to be co-localized with Rab7, a marker for late endosomes and lysosomes.

still under investigation. Evidence obtained from a neuronal cell line (N2a cells) transiently expressing CaV2.2 indicated that α2δ-1 does not affect the endocytosis of the channel [51]. Instead, α2δ subunits are suspected to control the trafficking of CaV channels either by promoting their transfer from the ER to the plasma membrane or by increasing their recycling.

The α2δ subunits are also synthesized by ER-bound ribosomes and translocated in the ER lumen. α2δ subunits are highly glycosylated [26,52] and this process is critical for the trafficking of CaV channels. It then appears obvious that, as the glycosylation state of α2δ 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 α2δ-1 and showed that, in addition to playing a role in stability/quality control and trafficking of α2δ-1, specific glycosylation sites of α2δ-1 are involved in the modulation of CaV1.2 biophysical properties [53].

The α2δ-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 α2δ-1 glycosylation maturation, trafficking, and cell surface expression. This LRP1/RAP complex also promotes the functional expression of CaV2.2 (cell surface expression and current density).

Besides glycosylation, α2δ is subject to additional post-translational modifications such as the formation of disulfide bonds and the proteolytic cleavage [56]. Disulfide bonds allow α2 to stay linked to δ and thus to the membrane after the proteolytic cleavage. The proteolytic cleavage of α2δ does not appear to affect the trafficking of CaV2 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 (CaV1.X and CaV2.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 CaV2.1 channels resulted in an increase in Ca2+ current.
amplitude [9]. It was hypothesized that β subunit binding to CaV2.1 α1 resulted in the masking of an ER retention motif present on the I–II loop [12,59]. However, studies performed on CaV1.2 and CaV2.2 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 CaV1.2 or CaV2.2 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 CaV1.2 or CaV2.2 to CaV3.1 α1 subunits, which do not require β subunits for their plasma membrane targeting, generated larger currents than wild type CaV3.1 [61,62]. Altogether, these studies support the existence of an ER export signal in the I–II loop of CaV1.2 and CaV2.2. Finally, extensive analysis of CaV1.2 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 CaV3.1 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 CaV2.1 and the other HVA channels [59–61]. For example, does this difference point to a CaV2.1 channel specificity? Further investigation will be needed to confirm this speculation.

β subunits increase the trafficking of CaV channels by playing the role of a trafficking switch but it was also shown that they can prevent the degradation of CaV channels by the proteasome [60,63]. In heterologous expression systems, β subunits reduce CaV1.2 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 CaV1.2 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 CaV1.2 trafficking to the plasma membrane was confirmed in hippocampal neurons [60]. Similarly, for CaV2.2 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 CaV2.2. This effect was blocked by proteasomal inhibitors [63]. It was later shown that the interaction with β subunits prevents the poly-ubiquitination of the CaV2.2 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 CaV1.2 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 CaV channel trafficking to the plasma membrane. In COS7 cells and rat dorsal root ganglion (DRG) neurons, Akt, a kinase in the PI3K/Akt 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 (CaV1.2 and CaV2.2) to the plasma membrane and an increase in calcium current density [67]. The effect of Akt on CaV1.2 was later shown to occur also in cardiomyocytes [68]. Altogether, these studies suggest that the phosphorylation of β2a promotes its chaperone role on CaV channels.

As we will discuss in the Endocytosis section of this review, G protein-coupled receptors (GPCRs) are potent modulators of CaV channel trafficking to the plasma membrane. In COS7 cells and rat dorsal root ganglion (DRG) neurons, Akt, a kinase in the PI3K/Akt 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 (CaV1.2 and CaV2.2) to the plasma membrane and an increase in calcium current density [67]. The effect of Akt on CaV1.2 was later shown to occur also in cardiomyocytes [68]. Altogether, these studies suggest that the phosphorylation of β2a promotes its chaperone role on CaV 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[L], 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 CaV2.2 [72]. The mutation of these consensus motifs in CaV2.2, 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 CaV2.2 in N2a cells and in DRG neurons. These findings demonstrate the functional involvement of the AP1 complex in the trafficking of CaV2.2 channels to the plasma membrane [72]. AP1 binding motifs are located in exon 37 of CaV2.2. 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 CaV2.2 channels containing exon 37a is higher than CaV2.2 channels containing exon 37b which reinforces the importance of this region for the trafficking of CaV2.2 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 CaV1.3, CaV1.4 and CaV2.1 (exon37a) which suggests that forward trafficking of these channels may also be AP1 dependent. Altogether, these data highlight the possibility that targeting AP1/CaV2.2 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 CaV2.2 channels in neurons by directly interacting with intracellular domains of CaV2.2, including its C-terminus [79]. In a recent study (using CaV2.2 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 CaV2.2 still has to be identified, it is possible that FMRP interferes with the binding of the API complex to the CaV2.2 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 CaV1.1 induces an increase in channel density in the plasma membrane and alters the kinetics of the CaV1.1-generated current in tsA-201 cells [83]. Stac proteins were also shown to alter the Ca2+-dependent inactivation of neuronal L-type channels Cav1.2 and Cav1.3, 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 (Cav2.1), Stac1 was shown to increase the expression of Cav3.2 [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 Ca2+-binding site in the pore**

Genes encoding CaVα1 subunits are transcribed into pre-messenger RNA that is subject to cell specific and developmentally regulated alternative splicing [73,87–92]. Splicing of CaVα1 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 CaV1.1, CaV1.2 and CaV2.1 have shown that truncated channels have physiological relevance by controlling the expression of full-length CaV channels [93–96]. Moreover, mutations that result in truncations of CaVα1 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 Cav1.2 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 Cav2.2 and Cav2.1 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 CaV2.1 and CaV2.2 in the trafficking of the channels to the plasma membrane [104]. This study shows that Ca2+-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 Ca2+ 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 Ca2+-dependent inactivation of CaV has been extensively studied [105]. However, CaM involvement in the trafficking of CaV remains unclear. CaM is able to bind several motifs in the C-terminus of Cav1.X and Cav2.X channels [105] and the deletion of these CaM binding motifs in Cav1.2 was shown to abolish the cell surface expression of the channels [13] and to alter Cav1.2 current amplitude [106–108] suggesting that CaM can modulate the trafficking of Cav1.2 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 Cav1.2 and examined the effect of CaM and a dominant negative CaM on its cell surface expression [109].
They quantified CaV1.2 plasma membrane expression by using fluorescence-activated cell sorting analysis. They did not observe an effect of CaM on CaV1.2 cell surface expression, thus concluding that CaM is not essential for the trafficking of CaV1.2 channels [109]. Overall, it can be argued that deletions and/or mutations in the C-terminus of CaV1.2 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 exercised when interpreting the results of such experiments.

Fully mature channels reach the plasma membrane as a protein complex formed by a main CaVα1 subunit and auxiliary subunits, glycosylated and associated with binding partners. These complexes are now able to play their physiological role in letting Ca2+ 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 CaV GPCRs**

GPCRs play critical roles in modulating the activity of CaV channels [4]. GPCRs have been described as part of signaling complexes together with CaVs, including CaV1.2 and β-2 adrenergic receptors [110,111], CaV2.1 and mGluR1 [112], CaV2.2 and opioid receptors, dopamine receptors (D1R and D2R), GABARβ 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 Gβγ and Gα. G protein-mediated regulation of CaV channels affects their biophysical properties [4,121–124]. For example, Ga(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 CaV channel activity. Indeed, Gβγ has been shown to directly interact with intracellular domains of the CaV2.2 family [126,127] and CaV3.2 channels [128,129]. For CaV2.2 channels, Gβγ interacts with the I–II loop and the N-terminus domain and it induces voltage-dependent inhibition [126,127]. For CaV3.2 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 CaV 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 CaV2.2 channels at the plasma membrane [72,114,116,117]. For D1R and ORL1, the interaction with CaV2.2 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 CaV2.2 [60] and/or the receptor itself could confer an additional trafficking motif to the channel complex. Moreover, a D2R-dependent increase in CaV2.2e37b 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 CaV2.2 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 CaV2.2, the internalization of the activated complex relies on both the AP2μ2 protein and an AP2 binding motif in the C-terminus of CaV2.2 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 CaV1.X and CaV2.X channels [8,135]. RGK proteins can utilize multiple mechanisms to inhibit CaV1.X and CaV2.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 CaV1.X and CaV2.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 CaV channels is still not fully understood.
The first evidence of an inhibitory effect of RGK proteins on CaV channels was presented by Béguin and colleagues [137]. These authors identified Gem as a binding partner for β subunits (β₁, β₂ and β₃) and then showed that the expression of Gem in Xenopus oocytes virtually abolished the currents generated by both CaV1.2 and CaV1.3 when co-expressed with β₁, β₂ or β₃ subunits. Finally, they correlated the reduction in Ca²⁺ current with a reduction in CaV1.2 cell surface expression. Indeed, they showed that the co-expression of Gem with CaV1.2/β-3 in HEK 293 cells prevents CaV1.2 channels from reaching the plasma membrane and this leads to the formation of intracellular channel aggregates. These results suggested that Gem competed with CaV channels localized to caveolae were inhibited, leaving CaV1.2 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 CaV? This could provide a powerful tool to tune synaptic transmission by targeting presynaptic CaV2.2 channels without affecting somatic CaV1.2 channels.

Other interactors affecting CaV channel endocytosis

While the forward trafficking effects of β subunits have been under continuous inquiry, whether β subunits have a role in endocytosis of CaV1.2 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 CaV1.2 in Xenopus oocytes through a dynamin-dependent interaction [147]. They later found that homodimerization of the β-SH3 domain was necessary for CaV1.2 endocytosis [148]. The endocytosis of CaV1.2 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 CaV, however further research is required to determine the net impact on CaV1.2 surface expression when considering the forward trafficking effect of full-length β subunits.

In hippocampal neurons, α-actinin, which binds to F-actin, was shown to stabilize CaV1.2 channels at the plasma membrane by preventing their endocytosis [149]. In resting conditions, α-actinin and apo-CaM (Ca²⁺-free CaM) both bind to site in the C-terminus domain of CaV1.2 (IQ CaM binding domain) [144,149,150]. During prolonged activity, the influx of Ca²⁺ increases the affinity of CaM for the C-terminus CaV1.2 and displaces the binding of α-actinin, thereby initiating the endocytosis of CaV1.2. Interestingly, the tumor suppressor eIF3e was shown to be responsible for a Ca²⁺-induced internalization of CaV1.2 [151]. It was then suggested that the displacement of α-actinin
from the CaV1.2 C-terminus could induce conformational changes that would allow eIF3e to bind to the intracellular II–III loop of CaV1.2 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 CaV1.2 C-terminus that can be unmasked when Ca2+ binds to apo-CaM [72].

The stromal interaction molecule 1 (STIM-1), the main activator of store-operated Ca2+ channels, was shown to directly interact with the C-terminus of CaV1.2 and reduce its plasma membrane density [152,153]. In hippocampal neurons, STIM-1 affects the depolarization-induced opening of CaV1.2 by both acutely inhibiting its gating and increasing its endocytosis via a dynamin-dependent mechanism [153]. The interaction STIM-1/CaV1.2 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 Dynoges and Pitstop, did not prevent the reduction in L-type current amplitude. Altogether, these studies suggest that STIM-1 can control CaV1.2 channel activity by different mechanisms depending on the intensity of the stimulus: for brief stimulation, STIM-1 reduces CaV1.2 activity, and for sustained stimuli, STIM-1 induces the internalization of the channels. Thus, STIM-1 provides an important negative feedback mechanism for Ca2+ influx.

As noted above, CaV1.2 and CaV2.2 channel surface expression is modulated by ubiquitination, leading to proteasomal degradation. CaV3.2 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 CaV3.2. Interestingly, USP5 is up-regulated in animal models of chronic pain and this up-regulation has been linked to the increase in CaV3.2 channel activity and its pro-nociceptive effect [155]. The exact mechanism of how the balance ubiquitination/de-ubiquitination of CaV3.2 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 CaV3.2 involves an endocytic mechanism. It is worth noting that the ubiquitination state of CaV3.2 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 CaV3.2/USP5 interaction affinity and then favors CaV3.2 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 CaV2.2 and to increase its cell surface expression [158,159]. The mechanism of action of CRMP2 on CaV2.2 has not yet been fully identified, but it may prevent CaV2.2 endocytosis as it does with NaV1.7 channels [160–163]. The effect of CRMP2 on CaV2.2 plasma membrane stability is modulated by post-translational modifications of CRMP2 like phosphorylation and SUMOylation [164,165].

The Ca2+ channel and chemotaxis receptor (cache) domain containing 1 protein (Cachd1), was shown to increase CaV2.2 cell surface expression in N2a cells and in hippocampal neurons [29]. Cachd1 protein was identified as an $\alpha_2-\delta$ like protein based on its structural homologies with $\alpha_2-\delta$s: it contains two Cache domains and a VWA domain although with a non-conserved MIDAS motif [166,167]. As opposed to $\alpha_2-\delta$ proteins which affect the forward trafficking of the channel, Cachd1 modifies CaV2.2 trafficking by reducing the rate of endocytosis of the channels [29]. Cachd1 protein was also shown to increase CaV3.1 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 CaV3.X channels was not investigated [168].

Functional and proteomic analyses of neuronal membranes have revealed a close proximity between CaV2.X channels and voltage- and Ca2+-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 CaV2.2 plasma membrane trafficking [30]. The co-expression of BK channels with CaV2.2/$\alpha_2-\delta-1$ induces the accumulation of CaV2.2 channels in Rab7-positive intracellular vesicles, a marker for late endosomes and lysosomes, suggesting that BK channels increase the internalization of CaV2.2 channels [30]. These results also suggest that the interaction with BK channels occurs only when the CaV2.2/$\alpha_2-\delta-1$ complex has reached the plasma membrane. Furthermore, the fact that BK channels outcompete CaV2.2 for the binding of $\alpha_2-\delta-1$ and increase CaV2.2 endocytosis is in favor of the idea that $\alpha_2-\delta-1$ has to remain associated with CaV2.2 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 CaVs. The auxiliary $\alpha_2-\delta-2$ subunit has been shown to be recycled by a Rab11-dependent pathway which controls CaV2.1 current density in tsA-201 cells [171]. In the cardiomyocyte cell line HL-1, CaV1.2 plasma membrane expression was shown to be dependent on the recycling
of the channel via a Rab11-dependent pathway [172]. However, in arterial smooth muscle cells, a Rab25-dependent pathway was shown to be involved in the recycling of CaV1.2 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 CaV1.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 CaV3.2 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 CaV3.2 channel expression by homocysteine [178]. This latter effect on the recycling of CaV3.2 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 CaV channel function [4], very few studies have reported effects on the trafficking of the channels.

Conclusion
The trafficking of CaV 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 CaV 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 CaV channels has greatly improved, gaps still remain. For example, how CaV 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 α,δ-2 recycling [171] but we have no experimental evidence whether neuronal CaV α1 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 CaV trafficking have been linked to pathological conditions such as neuropathic pain and ataxia, and deciphering the intricate mechanisms of CaV 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, Ca2+ channel and chemotaxis receptor domain containing 1 protein; cache, Ca2+ channel and chemotaxis receptor; CaM, calmodulin; CaV, voltage-gated Ca2+; 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; GHAS, 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; mIP3, 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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