The β-subunits of voltage-gated calcium channels regulate their functional expression and properties. Two mechanisms have been proposed for this, an effect on gating and an enhancement of expression. With respect to the effect on expression, β-subunits have been suggested to enhance trafficking by masking an unidentified endoplasmic reticulum (ER) retention signal. Here we have investigated whether, and how, β-subunits affect the level of CaV2.2 channels within somata and neurites of cultured sympathetic neurons. We have used YFP-CaV2.2 containing a mutation (W391A), that prevents binding of β-subunits to its I-I linker and found that expression of this channel was much reduced compared with WT CFP-CaV2.2 when both were expressed in the same neuron. This effect was particularly evident in neurites and growth cones. The difference between the levels of YFP-CaV2.2(W391A) and CFP-CaV2.2(WT) was lost in the absence of co-expressed β-subunits. Furthermore, the relative reduction of expression of CaV2.2(W391A) compared with the WT channel was reversed by exposure to two proteasome inhibitors, MG132 and lactacystin, particularly in neurites and growth cones. In conclusion, there is a marked effect of β-subunits on CaV2.2 expression, particularly in neurites, but our results point to protection from proteasomal degradation rather than masking of an ER retention signal.

The voltage-gated calcium channel (CaV) family plays a major role in the physiology of excitable cells. Three subfamilies of CaV channels have been identified; CaV1 to -3. The CaV1 (L-type) channels and the CaV2 (N-, P/Q-, and R-type) channels are thought to be heteromultimers composed of the pore-forming α1-subunit, associated with auxiliary CaV1, β- and α2δ-subunits (for reviews, see Refs. 1 and 2).

β-Subunits enhance the functional expression and influence the biophysical properties of the CaV1 and CaV2 channels, and two processes have been proposed to account for this. β-subunits hyperpolarize the voltage dependence of activation and increase the maximum open probability, which will increase current through individual channels and therefore result in augmented macroscopic current density (3–5). However, β-subunits have also been found to increase the number of channels inserted into the plasma membrane, as determined by gating charge measurements, imaging, and biochemical means (6–12). Nevertheless, increased membrane insertion of channels in the presence of a β-subunit has not been observed in all studies (13). As a mechanism for the effect on expression, β-subunits have been postulated to mask an ER retention signal in α1-subunits (9, 14), although no specific motif has been identified (14).

N-type calcium channels (CaV2.2) are present in both the central and peripheral nervous systems, and they have a major presynaptic role particularly in the sensory and autonomic nervous system in regulation of transmitter release (15–19). It remains unclear how CaV2 channels are trafficked into neuronal processes, although a previous study has implicated domains in the C terminus of the CaV2.2a long C-terminal splice variant (20). Here we have investigated whether and, if so, how β-subunits affect the expression and trafficking of N-type channels in cultured sympathetic neurons, particularly their penetration into neurites and growth cones. To do this, we have mutated tryptophan (Trp391) in the AID sequence in the I-II loop of CaV2.2. This residue is key to the interaction between β-subunits and the AID motif (21–24) and to the enhancement of functional expression of CaV2.2 by β-subunits (10, 25).

In this study, we found that YFP-CaV2.2(W391A) is expressed to a much smaller extent, compared with YFP- or CFP-CaV2.2(WT), particularly in the neurites and growth cones of SCG neurons. However, we did not find selective co-localization of YFP-CaV2.2(W391A) with an ER marker. In contrast, we found that the ratio of YFP-CaV2.2(W391A) to CFP-CaV2.2(WT) in both the neurites and somata was markedly increased by exposure to inhibitors of proteasomal degradation. This and further biochemical evidence suggests that the lack of high affinity interaction between a β-subunit and the I-I linker of CaV2.2(W391A) results in its increased proteasomal degradation relative to CaV2.2(WT) channel protein, rather than increased ER retention.
β-Subunit Regulation of Calcium Channel Degradation

EXPERIMENTAL PROCEDURES

Constructs Used—The calcium channel cDNAs used in this study were rabbit CaV2.2 (D14157), CaVβ1b(G61394), and α2δ-1 (M86621). These cDNAs were subcloned into the vector pRK5 for expression in SCGs. Other cDNAs used were mut3bgFP (26) in pMT2 vector, dsRed-ER (calreticulin, NM004343.3, in pdsRED2 vector), dsRed-Golgi (β1.4-galactosyltransferase, NM001497.3, in pdsRed2-C1 vector), pECFP, and pEYFP (Clontech).

Cell Culture and Heterologous Expression—The tsA-201 cells were cultured in DMEM, 10% FBS, 1% Glutamax, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). The cDNAs (all at 1 μg/μl) were transfected using Fugene6 (Roche Applied Science; DNA/Fugene6 ratio of 20 μg/30 μl). Unless otherwise stated, CaV2.2 constructs were co-expressed with α2δ-1 and β1b.

Western Blot Analysis—Whole cell lysates (2.5–250 μg of protein) were prepared from tsA-201 cells, as described for COS-7 cells (27), except for the inclusion of N-ethylmaleimide (240 mM). These and other samples were separated by SDS-PAGE on 3–8% Tris acetate or 12% BisTris gels and then transferred to polyvinylidene fluoride membranes. Immunodetection was performed with antibodies to the CaV2.2 II-III linker (Abcam), monoclonal anti-GFP Ab (Cell Signaling Technology), and monoclonal anti-ubiquitin Ab (P4D1; Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)).

Cell Surface Biotinylation—At 18 h after transfection, cells were rinsed twice with PBS and then incubated with PBS containing 1 mg/ml Sulfo-NHS-SS-Biotin (Perbio) for 30 min at room temperature (unless stated). The biotin solution was removed, and cells were rinsed once with PBS and twice with PBS containing 200 mM glycine at room temperature, to quench the reaction. The cells were then gently rinsed twice with PBS and then harvested in PBS containing protease inhibitors for 30 min on ice. The detergent lysates were then clarified by centrifugation (14,000 × g, 30 min, 4 °C). Biotinylated proteins were precipitated by adding 100 μl of streptavidin-agarose beads (Perbio) and incubated overnight at 4 °C. The streptavidin-agarose beads were washed three times and incubated for 1 h at 37 °C with 100 mM dithiothreitol and 2× Laemmli sample buffer. Eluted proteins were then resolved by SDS-PAGE.

Immunoprecipitation—YFP-CaV2.2 (WT or W391A mutant) was immunoprecipitated from transiently transfected tsA-201 cells as follows. Cells were harvested and lysed as described above. Clarified cell lysates were cleared with 50 μg of protein A-Sepharose (GE Healthcare) for 1 h at 4 °C. Supernatants were incubated with 2 μg/ml mouse monoclonal anti-GFP Ab (Clontech) overnight at 4 °C with constant agitation. A further 20 μg of protein A-Sepharose was added and incubated for 1 h at 4 °C. Beads were washed three times with PBS containing 0.1% Igepal and incubated for 15 min at 65 °C with 100 mM dithiothreitol and 2× Laemmli sample buffer. Eluted proteins were then resolved by SDS-PAGE.

Construction and Expression of GFP-tagged and Palmitoylated Constructs and Confocal Imaging—GFP was fused in-frame to the C terminus of CaVβ1b and expressed from the vector pMT2. The palmitoylation sequence MTLESIMACCL was added to the N terminus of the I-II loop (amino acids 356–483) of CaV2.2, and also to the CaV2.2 I-II loop containing the mutation W391A. GFP-tagged and palmitoylated constructs were transfected into tsA-201 cells in the ratio 1:2. Where constructs were missing, the volume was made up with blank vector (pMT2). Cells were fixed with 4% paraformaldehyde and stained with 4′,6-diamidino-2-phenylindole (DAPI) 42 h after transfection. Confocal microscopy was carried out using 1-μm optical slices.

Primary Culture of SCG Neurons—This was performed essentially as previously described (28). Rats were killed by either CO2 inhalation or cervical dislocation, according to United Kingdom Home Office Schedule 1 guidelines. SCGs were dissected from rats at postnatal day 17. Ganglia were desheathed and lightly gashed before successive collagenase (Sigma) and trypsin (Sigma) treatment, both at 3 mg/ml. To produce a single-cell suspension, ganglia were dissociated by trituration and centrifugation. Dissociated cells were plated onto glass-bottomed plates (MatTek Corp., Ashland, MA) pre-coated with laminin (Sigma), using one ganglion per five plates. Cells were maintained with Liebovic L-15 medium (Sigma), supplemented with 24 mM NaHCO3, 10% fetal bovine serum (Invitrogen), 33 mM glucose (Sigma), 20 mM l-glutamine, 1000 IU of penicillin, 1000 IU of streptomycin (Invitrogen), and 50 ng/ml nerve growth factor (NGF). MG132 (Sigma) or lactacystin (Sigma), when used, or their vehicle (DMSO) was applied following dilution in culture medium.

Microinjection—cDNAs were injected into SCG neurons 6–18 h after they were dissociated. Injection mixes consisted of YFP-CaV2.2 (WT or W391A) in pRK5, together with α2δ-1 and β1b, in a ratio of 3:2:2. Injection mixes for ratiometric analysis were composed of YFP-CaV2.2(WT or W391A) and CFP-CaV2.2(WT) together with α2δ-1 and β1b, in a ratio of 0.75:2.25:2.2. Injection mixes for Golgi and ER visualization were composed of GFP-Cav2.2(WT), α2δ-1, β1b, and dsRED-ER or dsRed-Golgi in a ratio of 3:2:2. Microinjection was performed with an Eppendorf micromanipulation system on a Zeiss Axiovert 200 m microscope, using the following settings: 90–120-hectopascal injection pressure, an injection time of 0.1–0.2 s, and constant pressure of between 40 and 50 hectopascals. The cDNA was injected at 25–50 ng/μl diluted in 200 mM KCl. For neurite intensity experiments, the injection mix was supplemented with 20 mM dextran 647 (Invitrogen).

Staining and Fixation—In some experiments, Cell Mask Deep Red plasma membrane stain (Invitrogen) was applied at a concentration of 2.5 μg/ml for 5 min in Hanks’ basal salt solution containing 47 mM sucrose. Neurons were then washed three times in Hanks’ basal salt solution containing sucrose and then fixed in 4% paraformaldehyde for 20 min. Neurons were washed a further three times in Hanks’ basal salt solution containing sucrose and then imaged.

Imaging—All imaging was performed on a Zeiss LSM 510 Meta scanning confocal microscope equipped with a Neofluor ×40/1.3 numerical aperture differential interference contrast.
oil immersion objective. The following imaging settings in nm were used throughout the project, independently and in combination. CFP imaging settings were as follows: emission band pass (BP), 475–525; laser 458 set to 17–25%; beam splitters, main dichroic 458/514 and secondary dichroic 545, GFP channel imaging settings were as follows: emission BP, 505–550; laser 488 set to 20–50%; beam splitters, main dichroic 488 and secondary dichroic 490. YFP channel imaging settings were as follows: emission BP, 530–600; laser 514 set to 3–9%; beam splitters, main dichroic 458/514 and secondary dichroic 545. Red channel imaging settings were as follows: emission BP, 560–615; laser 543 set to 40–60%; beam splitters, main dichroic 477/543 and secondary dichroic 490. Far red channel imaging settings were as follows: emission filters low pass, 650; laser 633 set to 30–60%; beam splitters, main dichroic UV/488/543/633.

Neurite Imaging—SCGs were imaged 18–24 h after microinjection, as stated. A neuron was centered and imaged using one or more of the above imaging channels. The pixel-dwell time was set to 3.20 μs, and the averaging was set to 4×. Settings were kept constant throughout each experiment to ensure comparison between conditions. For ratiometric comparisons were kept constant throughout each experiment to ensure

RESULTS

Expression and Properties of YFP-CaV2.2 and YFP-CaV2.2-(W391A)—In order to examine the trafficking of CaV2.2 in neurons, we made tagged constructs, attaching GFP, YFP, or CFP to the N terminus, for both the WT and the W391A mutant CaV2.2. We first examined the stability of these constructs by immunoblot following expression in tsA-201 cells. No free YFP or CFP was observed (supplemental Fig. 1, A and B), indicating that the fusion proteins were intact, as described previously for GFP-CaV2.2 (27). We then compared the properties of YFP-CaV2.2 and YFP-CaV2.2(W391A), together with the accessory subunits αδ1 and β1b, expressed in Xenopus oocytes. As expected, the W391A mutation reduced Ito very substantially (supplemental Fig. 1C), by 81% at −5 mV and by 73% at 0 mV (supplemental Fig. 1D). This mutation also depolarized both the activation and steady-state inactivation curves, as expected for the absence of interaction of the I-II linker with β-subunits (supplemental Fig. 1, C and E). Similar results were obtained previously in tsA-201 cells for the non-tagged channels (10), where an 81% reduction in peak current density was observed for untagged CaV2.2(W3891A) compared with the WT channel.

Importantly for our subsequent studies, co-expression of YFP-CaV2.2 with YFP-CaV2.2(W391A) did not cause any significant suppression of CaV2.2 currents (supplemental Fig. 1D), unlike the dominant negative suppression that we observed.
previously to result from expression of CaV2.2 together with non-functional truncated constructs (27, 31, 32).

No Interaction Was Observed between GFP-tagged CaVβ1b and the I-II Loop of CaV2.2(W391A)—In order to examine further whether the small currents arising from CaV2.2(W391A) were due to plasma membrane expression, despite lack of interaction with β-subunits, or to a low affinity interaction of the mutant I-II linker with β-subunits, we devised an imaging assay to specifically examine this interaction.

When GFP-tagged β1b was expressed alone in tsA-201 cells, it showed a uniform distribution throughout the cytoplasm and was also present in the nucleus (Fig. 1A). We took the I-II loop (amino acids 356–483) of CaV2.2 and added a palmitoylation sequence, MTLESIMACCL, to its N terminus (palm CaV2.2 I-II), in order to target it to the plasma membrane. We found that co-expression of palmitoylated CaV2.2 I-II with GFP-tagged CaVβ1b directed GFP-CaVβ1b out of the nucleus to the plasma membrane (Fig. 1B), demonstrating a positive interaction. In
contrast, in the presence of palmitoylated I-II loop containing the W391A mutation (palm CaV2.2 I-II W391A), the GFP-β1b still showed a uniform distribution throughout the cytoplasm and in the nucleus (Fig. 1C). The inset schematic (in Fig. 1D) shows the likely mechanism for membrane association of GFP-β1b illustrated in Fig. 1B. Quantification of line scans, including those shown in Fig. 1D, indicated that there was no difference between the ratio of nuclear to membrane staining for GFP-β1b alone and GFP-β1b expressed with palmitoylated CaV2.2 I-II W391A, whereas in the presence of the WT CaV2.2 I-II loop construct, the ratio was more than 14-fold greater than for CaV2.2 I-II W391A (Fig. 1E). This confirms the complete lack of interaction of β1b-subunit with the CaV2.2 I-II linker containing the W391A mutation.

Quantification of Expression of YFP-CaV2.2 and YFP-CaV2.2(W391A) in SCG Neurites—Following their microinjection into cultured SCG neurons, both YFP-CaV2.2(WT) and YFP-CaV2.2(W391A), in combination with α2δ-1 and β1b, resulted in expression in both the somata and the neurites (Fig. 2A). We developed an assay to examine quantitatively the amount of fluorescence in the neurites, to determine if there was any difference in this compartment between the expression of YFP-CaV2.2 and YFP-CaV2.2(W391A). We imaged the entire neurite arborization and excluded fluorescence from the soma (Fig. 2B). Cells were injected after 6 h in culture and imaged 18 h after microinjection. We then determined the total neurite area, using dextran 647, to obtain the neurite fluorescence density for each condition (see “Experimental Procedures”). The total neurite area of injected SCG neurons was not altered under the different conditions (Fig. 2C), but the fluorescence density was significantly reduced by 51% for YFP-CaV2.2(W391A), compared with YFP-CaV2.2 (Fig. 2D).

To examine the possibility that YFP-CaV2.2 was trafficked to the plasma membrane within the soma, which then extended neurites containing these channels, we also microinjected cells after 24 h in culture, when the neurites were already very extensive, and imaged them 24 h later. We found that the differential between YFP-CaV2.2(W391A) and YFP-CaV2.2 was maintained under this condition (Fig. 2D), with a 51% reduction in neurite fluorescence density for the YFP-CaV2.2(W391A) construct, suggesting that the channels reached the neurites, at least in part, on internal membranes.

In order to determine whether the reduction of expression of YFP-CaV2.2(W391A) in the neurites occurred as a result of retention of the mutant channels in the cell body, we imaged the expression in the somatic compartment, in cells injected after 6 h in culture, and imaged 18 h after microinjection. The somatic fluorescence density was quite variable between neurons, being 169.1 ± 49.1 arbitrary units/μm² (n = 10) for YFP-CaV2.2(WT) and 116.0 ± 34.0 arbitrary units/μm² for YFP...
CaV2.2(W391A) (n = 8; p > 0.05). Nevertheless, these results do not provide any evidence for selective retention of the mutant channels within the cell body as a mechanism for the reduction in their fluorescence within the neurite compartment.

The Role of β-Subunits in the Expression of YFP-CaV2.2 and YFP-CaV2.2(W391A) in SCG Neurites—Because we observed variability of expression levels between different neurons, we then included CFP-CaV2.2 in each condition, in order to have an internal control, rather than comparing between neurons (Fig. 3, A and B). In each experiment, confocal settings were used such that the control ratio of WT YFP-CaV2.2/CFP-CaV2.2 fluorescence was approximately unity. Other experimental conditions were then compared with this (Fig. 3, A and B). Using this assay, we quantified the effect of expression of the W391A mutant channel, by determining the ratio of YFP-CaV2.2(W391A)/CFP-CaV2.2 fluorescence in the cell bodies alone (Fig. 3A) or in the total neurite compartment excluding the soma (Fig. 3B). The results show that there was a reduction in the expression of YFP-CaV2.2(W391A) relative to WT CaV2.2 of 63.2% in the somatic compartment (Fig. 3C) and a more marked reduction of 77.7% in the total neurite compartment (Fig. 3D), determined by this method.

We then investigated the effect on the relative expression of YFP-CaV2.2(W391A) compared with CFP-CaV2.2(WT) of manipulating the concentration of β-subunits, together with the additional presence of a CaV2.2 I–II linker construct to sequester endogenous β-subunits. The results demonstrate the dependence of expression of WT CFP-CaV2.2 relative to YFP-CaV2.2(W391A) on both exogenous and endogenous β-subunits (Fig. 3E). The ratio between CaV2.2(W391A) and CaV2.2(WT) increased, particularly when exogenous β1b was omitted, indicating that β-subunits are a limiting factor in the expression of WT CaV2.2 in the neurites. In agreement with this, we also observed that the expression of CaV2.2(WT) in tSA-201 cells was reduced by 35% in the absence of β-subunit co-expression, whereas no effect was observed on the expression of CaV2.2(W391A) (supplemental Fig. 2, A and B). Furthermore, the effect of a reduction in β-subunit co-expression on the level of YFP-CaV2.2(WT) in neurites was also observed directly, without using the ratiometric method (supplemental Fig. 2C).

Taken together, these results indicate that YFP-CaV2.2(W391A) is expressed in neurites to a significantly smaller extent than YFP-CaV2.2 or CFP-CaV2.2, and its level of expression is not dependent on β-subunits, whereas the expression of WT CaV2.2 is strongly dependent on the presence of β-subunits.

Subcellular Localization of YFP-CaV2.2 and YFP-CaV2.2(W391A) in SCG Neurites—The results described above suggested to us that the low concentration of YFP-CaV2.2(W391A) that is present in the neurites may not be associated with the plasma membrane. To test the accepted view that the role of β-subunits is to mask an ER retention signal (9), we compared the localization of the channels in the ER compared with post-ER compartments. To do this, we concentrated particularly on growth cones because we found the ER to be present not only throughout the soma, where it co-localized with GFP-CaV2.2(WT) (supplemental Fig. 3A), but also as a continuous network within the neurites, as previously described for hippocampal neurons (33). However, ER staining extended only into the bulb of the growth cone and was not present in the lamellipodia (Fig. 4A). A similar distribution was found for a Golgi marker in the growth cone bulb (supplemental Fig. 3B), although overall it showed a more restricted localization than the ER marker.

We found that WT GFP-CaV2.2 was concentrated in the bulb of the growth cones, but was also distributed in the filopodia and lamellipodia (Fig. 4A). We then compared the co-localization of GFP-CaV2.2(W391A) and GFP-CaV2.2(WT) with the ER marker dsRed-ER in the growth cones (Fig. 4, A and B). There was no differential retention of GFP-CaV2.2(W391A) within the ER region in the growth cone bulb because the ratio of fluorescence for GFP-CaV2.2(W391A) compared with GFP-CaV2.2(WT) was 61.7% within the ER region and 55.2% for the lamellipodia. There was no difference in the fluorescence signal for the ER marker under the two different conditions (supplemental Fig. 3C). To confirm this finding, we then determined the ratio of YFP/CFP fluorescence in both the bulb and the lamellipodia regions of growth cones for cells co-expressing CFP-CaV2.2(WT) and either YFP-CaV2.2(WT) (Fig. 5A) or YFP-CaV2.2(W391A) (Fig. 5B). Quantification of these data showed a reduction of YFP-CaV2.2(W391A) relative to the WT channel by 74.7% in the growth cone bulb region (Fig. 5C) and a slightly larger decrease, by 79.6% in the lamellipodia (Fig. 5D). These results do not provide evidence that CaV2.2(W391A) is selectively retained in the ER compartment.

Retrograde Transport of YFP-CaV2.2 but Not YFP-CaV2.2(W391A) in SCG Neurites—We next examined whether either YFP-CaV2.2(WT) or YFP-CaV2.2(W391A) within the neurites could be observed as discrete motile particles, as well as diffuse fluorescence. In neurons that had been injected 6 h previously, it was possible to observe particles within neurites containing YFP-CaV2.2(WT), and we therefore performed time series experiments to determine whether they were motile (supplemental Fig. 4 and associated movie). Almost all of the motile particles were observed to move in a retrograde direction and contained both YFP-CaV2.2 and CFP-CaV2.2 when these were co-expressed (Fig. 6A shows time series as kymographs). The average rate of movement of these particles was 0.54 ± 0.04 μm/s (n = 9), with a maximum rate of 1.17 ± 0.09 μm/s (n = 9). When a YFP-TrkA receptor construct was co-expressed, it was observed to co-localize in the motile particles (data not shown), indicating that they derive at least in part from growth cones. In contrast, when YFP-CaV2.2(W391A) was co-expressed with CFP-CaV2.2(WT), very little co-localization of YFP-CaV2.2(W391A) with CFP-CaV2.2(WT) (12.0%) was observed in the retrogradely motile particles (Fig. 6, B and C). These results do not support the hypothesis that there was a greater retrograde transport of the mutant channel as an explanation for its lower level in the neurites.

Taking our ratiometric imaging experiments together, we found that the ratio of YFP-CaV2.2(W391A) to CFP-CaV2.2(WT) was 36.8% of the ratio of the WT channels in the soma, falling to 25.3% in the growth cone bulb, 20.4% in the
lamellipodia, and 12.0% in retrograde particles, indicating that there is a gradient from the soma to distal structures (Fig. 6D).

Proteasomal Degradation of YFP-CaV2.2 and YFP-CaV2.2(W391A) in SCG Neurites—The results described above suggested that YFP-CaV2.2(W391A) was subjected to increasing loss relative to the WT channel, at increasing distances from the soma. A potential explanation for this observation is that the mutant channels that do not interact with the \( \beta\)-subunit have a shorter lifetime. This possibility was also suggested by our observation that when \( \beta\)-subunits were not co-transfected with

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**FIGURE 3.** Expression of WT and W391A mutant YFP-CaV2.2 in SCG neurons, using CFP-CaV2.2 as an internal control. A, examples of SCG neuron somata expressing CFP-CaV2.2(WT) (left) together with YFP-CaV2.2(WT) (top right) or YFP-CaV2.2(W391A) (bottom right), injected after 6 h in culture, and imaged 18 h later. Scale bar, 20 \( \mu m \). B, examples of SCG neurons expressing CFP-CaV2.2(WT) (left), together with YFP-CaV2.2(WT) (top right) or YFP-CaV2.2(W391A) (bottom right), injected after 6 h in culture, and imaged 18 h later. Scale bar, 100 \( \mu m \). C, bar chart of the ratio of YFP/CFP fluorescence in somata from data such as those in A, for YFP-CaV2.2(WT) (black bar, \( n = 5 \)) and YFP-CaV2.2(W391A) (white bar, \( n = 6 \)), both expressed together with CFP-CaV2.2. The statistical significance between the two conditions is shown: ***, \( p < 0.0001 \), Student’s t test. D, bar chart of the ratio of YFP/CFP fluorescence in neurites, excluding the cell bodies, from data such as those in B, for YFP-CaV2.2(WT) (black bar, \( n = 6 \)) and YFP-CaV2.2(W391A) (white bar, \( n = 7 \)), both expressed together with CFP-CaV2.2. The statistical significance between the two conditions is shown: ***, \( p < 0.0001 \), Student’s t test. E, bar chart of the ratio of YFP/CFP fluorescence, from data such as those in B, for YFP-CaV2.2(W391A) expressed together with CFP-CaV2.2, in conjunction with the standard concentration of \( \beta\)Ib (black bar, \( n = 10 \)) with a 20-fold dilution of \( \beta\)Ib (gray bar, \( n = 13 \)), with no exogenous \( \beta\)-subunit (hatched bar, \( n = 10 \)), and with the I-II linker of CaV2.2 (white bar, \( n = 11 \)). The statistical significances between the conditions are shown as follows: ***, \( p < 0.001 \), one-way analysis of variance and Tukey’s post hoc test. Error bars, S.E.
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\text{CaV}_2.2\text{ in tsA-201 cells, a reduced level of CaV}_{2.2}\text{ protein was observed (supplemental Fig. 2, A and B).}$

We therefore examined whether increased degradation of YFP-CaV$_2.2$(W391A) compared with GFP-CaV$_2.2$(WT) could be responsible for its reduced level in SCG neurites and growth cones. To do this, we applied the proteasome inhibitor MG132 (34, 35) for 18 h, from 30 min after transfection, at concentrations of 250 nM. We found that the ratio of YFP-CaV$_2.2$(W391A) to GFP-CaV$_2.2$(WT) showed a concentration-dependent increase, from 0.28 in the absence of MG132 to 1.0 in the presence of 250 nM MG132 (p < 0.001; Fig. 7C). Furthermore, there was no effect of MG132 on the overall neurite morphology as measured by neurite branching (supplemental Fig. 5B).

The total neurite compartment, we found that the ratio of YFP-CaV$_2.2$(W391A) to CFP-CaV$_2.2$(WT) also showed a concentration-dependent increase, from 0.28 in the absence of MG132 to 0.55 in the presence of 1 μM MG132 (p < 0.001; Fig. 7C). Furthermore, there was no effect of MG132 on the overall neurite morphology as measured by neurite branching (supplemental Fig. 5B).

In order to ascertain whether this effect would generalize to other proteasome inhibitors, we also used lactacystin (10 μM). We found that this proteasome inhibitor also markedly increased the ratio of YFP-CaV$_2.2$(W391A) to CFP-CaV$_2.2$(WT) in the SCG cell bodies (Fig. 7D and E). In these experiments, the SCG somata were all imaged in the plane of the nucleus, and lactacystin can be seen to increase the perinuclear CaV$_2.2$ fluorescence concentration.

**FIGURE 4. Comparison of co-localization of WT and W391A mutant GFP-CaV$_2.2$ with ER marker in neurites and growth cones.** A, images of SCG growth cones showing the expression of GFP-CaV$_2.2$(WT) (top left) and GFP-CaV$_2.2$(W391A) (bottom left), compared with the distribution of the subcellular organelle marker, dsRed-ER (center). The merged images are shown on the right, and the extents of the growth cones are identified by a dotted white line, determined by the use of Cell Mask dye. Scale bars, 20 μm. The black cross represents the ER region (100% ER signal), and the white cross represents the lamellipodia region outside the ER marker but within Cell Mask stain region. B, bar chart of GFP fluorescence in a region of interest (ROI) either in the region of high ER staining, indicated by the black cross (left pair of bars), or in the extents of growth cones, indicated by the white cross (right pair of bars), from data such as that in A, for GFP-CaV$_2.2$(WT) (black bars; n = 20) and GFP-CaV$_2.2$(W391A) (white bars; n = 21). The statistical significance between the two conditions is shown: **, p = 0.004; *, p = 0.013; Student’s t test. Error bars, S.E.
DISCUSSION

In this study, we initially investigated the role of β-subunits on the distribution of CaV2.2 channels in the cell bodies, neurites, and growth cones of SCG neurons. The β-subunits bind with high affinity to the AID sequence within the I-II linker of all CaV1 and CaV2β1-subunits (21). The 18-amino acid AID motif has a conserved Trp that is essential for binding β-subunits (10, 21, 42). Structural data have provided detailed information concerning the interaction between the AID motif and CaVβ complex, showing that this Trp is embedded in the AID binding groove within the guanylate kinase domain of β-subunits (22–24, 43). One of the main effects of β-subunits is to increase current density for all CaV1 and CaV2 calcium channels. The mechanism for this increase is thought to be a result of both greater insertion into the plasma membrane (6–12) and hyperpolarization of the voltage-dependence of channel activation and increase in the maximum open probability (3, 4, 13).

Although the relative importance of increased membrane insertion has been disputed (13), we found previously in tsA-201 cells that when β-subunits were not co-expressed with WT CaV2.2 channels or when a β-subunit was co-expressed with CaV2.2(W391A) channels, there was reduced expression at the plasma membrane, as determined by cell surface biotinylation, which would be expected to contribute to the reduced currents.

FIGURE 5. Comparison of co-localization of WT and W391A mutant CFP/YFP-CaV2.2 in growth cones. A and B, examples of growth cones from SCG neurons expressing CFP-CaV2.2(WT) (top panels) and either YFP-CaV2.2 (A, bottom) or YFP-CaV2.2(W391A) (B, bottom), injected after 6 h in culture, and imaged 18 h later. ROIs such as those shown here, in the bulb of the growth cone (red cross) and in the lamellipodia (white cross), were used to calculate the data in C and D. Scale bars, 20 μm. C and D, bar chart of the ratio of YFP/CFP fluorescence in a ROI in the ER region (C) or in the lamellipodia region in the extremities of growth cones (D), from data such as those in A and B, for YFP-CaV2.2(WT) (black bars; n = 10) and YFP-CaV2.2(W391A) (white bars; n = 11), expressed together with CFP-CaV2.2. The statistical significance between the two conditions is shown: ***, p < 0.0001, Student’s t test. Error bars, S.E.
observed. These results therefore provided strong evidence that the binding of $\beta$-subunits to these channels is an important requirement for functional expression of CaV2.2 at the plasma membrane (10). Similar results were also obtained previously for CaV1.2 channels (11).

However, we observed in Xenopus oocytes (present study) and previously in tsA-201 cells (10) that when CaV2.2(W391A) channels were expressed together with a $\beta$-subunit, small currents remained, either because the overexpressed $\beta$-subunit was able to bind with very low affinity to the mutated I-II linker of CaV2.2(W391A) or to other domains of the channel or because, in the absence of interaction with exogenous $\beta$-subunit, the mutant channel is still able to traffic to a small extent to the plasma membrane and conduct current. Furthermore, currents through CaV2.2(W391A) channels show a depolarized activation and steady-state inactivation (supplemental Fig. 1, C and E), characteristic of lack of interaction with a $\beta$-subunit (10). The reduced level of CaV2.2(W391A) channels at the cell surface could be due to reduced forward trafficking (9), increased endocytosis, or increased degradation from an intracellular compartment. In the present study, we have addressed these possibilities, particularly with respect to expression of the channels in the neurites of SCG neurons.

A previous study showed that $\beta$-subunit interaction with CaV1.2 was essential for trafficking into dendritic spines in hippocampal neurons (25). However, for the N-type channel CaV2.2, it is not yet possible to study its plasma membrane localization by imaging techniques because of the absence of a functional CaV2.2 construct with an exofacial tag and the lack of antibodies to extracellular loops.

In the present study, we have found that both XFP-CaV2.2(WT) and XFP-CaV2.2(W391A) channels are well expressed following microinjection into SCG neuronal somata. However, there was a lower level of YFP-CaV2.2(W391A) compared with YFP-CaV2.2(WT), and this was most pronounced in neurites and in their growth cones. These experiments benefited from the use of the ratiometric assay, in which the ratio of YFP-CaV2.2(W391A) to CFP-CaV2.2(WT) was compared between neurons in the same experiment with the ratio of YFP-CaV2.2(WT) to CFP-CaV2.2(WT). Using this technique, differences due to variation in microinjection efficiency or different expression levels are eliminated. In this way, we observed that, whereas the penetration of YFP-CaV2.2(WT) into the neurites was strongly dependent on the presence of $\beta$-subunits, the level being reduced by up to 70% in their absence, there was no similar effect on YFP-CaV2.2(W391A) channel expression.

![FIGURE 6. Ratiometric analysis of retrogradely moving particles in neurites of SCG neurons expressing CFP-CaV2.2(WT) and YFP-CaV2.2(WT/W391A). A and B, CFP-CaV2.2(WT) (top row) and YFP-CaV2.2(WT) (A) or YFP-Cav2.2(W391A) (B) (middle row) were co-expressed in SCG neurons, and retrogradely moving particles were imaged, 6 h after microinjection. Time series were recorded in the CFP and YFP channels. Imaging sensitivity was balanced in the CFP and YFP channels. Image stacks were corrected for non-motile structures, by subtraction of the time series median image. Kymographs were plotted along particle trajectories, showing time in the vertical direction, and the merged image is shown in the bottom row. The intensity of CFP-containing particles was measured and compared with the same ROI in the YFP channel. No particle movement was observed in the YFP channel of the YFP-CaV2.2(W391A)/CFP-CaV2.2(WT) condition (B). Scale bar, 20 $\mu$m. Vertical time scale, 75 s. C, bar chart of the ratio of YFP/CFP fluorescence in retrograde particle ROIs, from data such as those in A and B, for YFP-CaV2.2(WT) (black bar; $n = 6$ neurons) and YFP-CaV2.2(W391A) (white bar; $n = 6$ neurons), expressed together with CFP-CaV2.2(WT). The statistical significance between the two conditions is shown: ***, $p < 0.001$, Student’s t test. D, diagram of the observed gradient of YFP-CaV2.2(W391A) relative to CFP-CaV2.2 from the soma to the growth cones and retrogradely moving particles.](#)
FIGURE 7. Effect of proteasomal inhibition by MG132 and lactacystin on expression of YFP-tagged WT and W391A-CaV2.2 in SCG somata and neurites, using CFP-CaV2.2 as an internal control. A, examples of SCG neuron somata expressing CFP-CaV2.2(WT) (left), together with YFP-CaV2.2(W391A) (right), injected after 6 h in culture, and imaged 18 h later, in the presence of 50 nM (top), 500 nM (middle), and 1 μM (bottom) MG132. Scale bars, 20 μm. Note that the image plane does not go through the nucleus in all cases. B, bar chart of the ratio of YFP/CFP fluorescence in cell bodies, from data such as those in A, for YFP-CaV2.2(WT) (black bar; n = 14), YFP-CaV2.2(W391A) (white bar; n = 12), and YFP-CaV2.2(W391A) together with 50 nM (light gray bar; n = 13), 250 nM (dark gray bar; n = 13), or 1 μM (hatched bar; n = 13) MG132. All experiments also included CFP-CaV2.2(WT). The statistical significance between YFP-CaV2.2(W391A) in the absence and presence of MG132 is shown: * p < 0.05, one-way analysis of variance and Bonferroni’s post-test. C, bar chart of the ratio of YFP/CFP fluorescence in neurites, for YFP-CaV2.2(WT) (black bar; n = 17), YFP-CaV2.2(W391A) (white bar; n = 17), and YFP-CaV2.2(W391A) together with 50 nM (light gray bar; n = 13), 250 nM (dark gray bar; n = 14), or 1 μM (hatched bar; n = 19) MG132. All experiments also included CFP-CaV2.2(WT). The statistical significances are shown as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.001, one-way ANOVA and Bonferroni’s post-test. D, examples of SCG neuron somata expressing CFP-CaV2.2(WT) (left), together with YFP-CaV2.2(W391A) or YFP-CaV2.2(WT) (right), injected after 6 h in culture, and imaged 18 h later, in the presence of DMSO or lactacystin (10 μM), as indicated. Scale bars, 20 μm. Note that the image plane goes through the nucleus in all cases. E, bar chart of the ratio of YFP/CFP fluorescence in cell bodies, from data such as those in D, for YFP-CaV2.2(WT) + DMSO (black bar; n = 8), CaV2.2(WT) + lactacystin (white bar; n = 7), YFP-CaV2.2(W391A) + DMSO (light gray bar; n = 11), and CaV2.2(W391A) + lactacystin (dark gray bar; n = 11). All experiments also included CFP-CaV2.2(WT). The statistical significances are shown: ***, p < 0.001; **, p < 0.01, one-way analysis of variance and Bonferroni’s post-test. Error bars, S.E.
Because it has been postulated that the mechanism of action of \( \beta \)-subunits is to mask an ER retention signal (9, 14), we investigated whether YFP-Ca\(_{\text{a}2.2}\)(W391A) was retained within the neuronal somata, where ER retention might be particularly expected to occur. We found that there was no selective retention of YFP-Ca\(_{\text{a}2.2}\)(W391A) compared with CFP-Ca\(_{\text{a}2.2}\)(WT) in the cell soma, indicating that this was not an explanation for its lack of expression in the neurites. We found that the ER was present throughout the SCG neurites but only extended into the bulb of the growth cones. Because YFP-Ca\(_{\text{a}2.2}\)(W391A) fluorescence in the neurites was largely diffuse rather than confined to discrete organelles, it was therefore possible that much of the YFP-Ca\(_{\text{a}2.2}\)(W391A) that exists in the neurites might be present within the ER. However, no evidence was obtained for selective ER retention of the mutant Ca\(_{\text{a}2.2}\)(W391A) channel because the ratio of fluorescence of the mutant compared with the wild-type channel was very similar in the ER-rich region within the bulb of the growth cone, compared with the lamellipodia region, where ER was absent.

Endogenous N-type channels have been observed in growth cones of cultured sympathetic neurons (44). Although we have no direct evidence that YFP-Ca\(_{\text{a}2.2}\)(WT) reached the plasma membrane of the neurites when expressed in SCG neurons, we have indirect evidence that this is the case. We have observed retrograde transport in neurites of particles in which YFP-Ca\(_{\text{a}2.2}\)(WT) and CFP-Ca\(_{\text{a}2.2}\)(WT) are co-localized and have also observed co-localization of these particles with TrkA receptors (data not shown), which are internalized following binding to NGF (45) and therefore originate from the plasma membrane. Almost no retrograde transport of YFP-Ca\(_{\text{a}2.2}\)(W391A) was observed, suggesting that it only reached the plasma membrane to a very small extent and that increased endocytosis and retrograde transport was not an explanation for its lower levels in neurites and growth cones. Furthermore, we noted that there was a gradient in the ratio of YFP-Ca\(_{\text{a}2.2}\)(W391A) to CFP-Ca\(_{\text{a}2.2}\)(WT) relative to the ratio of the YFP- and CFP-WT channel pair from the soma, where it was 36.8%, decreasing to 12.0% in retrograde particles, suggesting that as it progresses down the neurites, the YFP-Ca\(_{\text{a}2.2}\)(W391A) is subjected to increasing loss or degradation relative to the WT channel (Fig. 6D).

In agreement with this hypothesis, we found that the ratio of YFP-Ca\(_{\text{a}2.2}\)(W391A) to CFP-Ca\(_{\text{a}2.2}\)(WT) in both somata and neurites was markedly increased by exposure to a proteasome inhibitor (MG132) in a concentration-dependent manner. This result was replicated with another proteasome inhibitor (lactacystin). In addition, the total fluorescence attributable to both YFP-Ca\(_{\text{a}2.2}\)(W391A) and CFP-Ca\(_{\text{a}2.2}\)(WT) was increased by MG132 in the soma, indicating that the change in ratio is a result of reduced degradation. Our study is in agreement with a report in abstract form that Ca\(_{\text{a}2.1,2}\) is a substrate for proteasomal degradation and is protected by the \( \beta \)-subunit (46). Components of the ubiquitination machinery and of the proteasome have been identified in axons and growth cones (47–49), and it is possible that the proteasome inhibitors act in neurites as well as in the somata to inhibit the degradation of YFP-Ca\(_{\text{a}2.2}\)(W391A), which is otherwise degraded more rapidly than its WT counterpart, due to protection of the WT channel.

**FIGURE 8.** Effect of proteasomal inhibition by MG132 on expression of WT and W391A-Ca\(_{\text{a}2.2}\) in tsA-201 cells. A, cell surface biotinylation experiment, showing biotinylated Ca\(_{\text{a}2.2}\) (top) and total Ca\(_{\text{a}2.2}\) (middle), for cells transfected with Ca\(_{\text{a}2.2}\)(WT)/Ca\(_{\text{a}2.2}\)(W391A) (lanes 1 and 2) and Ca\(_{\text{a}2.2}\)(WT)/Ca\(_{\text{a}2.2}\)(W391A) (lanes 3 and 4) either treated with vehicle DMSO (lanes 1 and 3) or MG132 (250 nm, lanes 2 and 4). Results are representative of nine experiments with similar results. GAPDH was used as a loading control (bottom). The biotinylation procedure did not biotinylate any cytoplasmic protein (Akt) (supplemental Fig. 6A). B, bar chart showing proportion of total Ca\(_{\text{a}2.2}\) present at the cell surface from nine experiments, including that illustrated in Fig. 8A, for Ca\(_{\text{a}2.2}\)(WT)/Ca\(_{\text{a}2.2}\)(W391A) (lanes 1 and 2) and Ca\(_{\text{a}2.2}\)(WT)/Ca\(_{\text{a}2.2}\)(W391A) (lanes 3 and 4) either treated with vehicle DMSO (lanes 1 and 3) or MG132 (250 nm, lanes 2 and 4). Results were corrected with the loading control (GAPDH). **, p < 0.01; ***, p < 0.001, one-way analysis of variance and Bonferroni’s post-test. C, bar chart of total Ca\(_{\text{a}2.2}\) from eight experiments, including that illustrated in Fig. 8A, expressed as a ratio of that in the presence and absence of MG132 for Ca\(_{\text{a}2.2}\)(WT)/Ca\(_{\text{a}2.2}\)(W391A) (black bar) and Ca\(_{\text{a}2.2}\)(WT)/Ca\(_{\text{a}2.2}\)(W391A) (white bar). **, p = 0.00026, Student’s t test. Error bars, S.E.
by interaction with a β-subunit. Thus, the proteasome inhibitor normalizes the expression levels of the mutant and WT channels. Despite this apparent rescue of Ca_{v}2.2(W391A) by the proteasomal inhibitor, this is likely to represent the build-up of intracellular polyubiquitinated, rather than functional, channels. Indeed, our results show that MG132 did not enhance the cell surface expression of Ca_{v}2.2(W391A) in tsA-201 cells but very markedly increased its observed level of ubiquitination.

Evidence suggests that interaction of β-subunits via the AID region may promote lower affinity interactions with other domains of the channel (10, 50). Interference by the β-subunit with the proteasomal degradation of the channel may thus involve the β-subunit promoting correct channel folding and masking ubiquitination sites either on the I-II linker or elsewhere on the channel, but it may also involve prevention of retrotranslocation out of the ER (for a review, see Ref. 51). It is also possible that the β-subunit may play a direct, rather than indirect, role in inhibiting these processes.

In conclusion, interaction with the β-subunit plays an essential role in the stabilization and hence functional expression of Ca_{v}2.2 channels in addition to effects on channel biophysical properties studied previously (3–5, 10). Our studies suggest that the mechanism for this effect is for the most part not the promotion of forward trafficking as a result of decreased ER retention of the channels following interaction with β-subunit but rather protection from proteosomal degradation. We have found that loss of high affinity interaction with a β-subunit via the I-II linker results in reduced expression of Ca_{v}2.2(W391A), particularly in neurites and growth cones. This opens an important and novel area for investigation of the dynamics of calcium channel regulation, for example in the control of neuropathic pain, where N-type channel function is up-regulated (52). This is associated with an up-regulation and increased trafficking of α_2δ-1 subunit protein (53). However it also involves interaction with β-subunits because knock-out of β_2-subunits, the main β-subtype in sensory neurons, results in a reduction of Ca_{v}2.2 channels and a phenotype including a reduction in late phase pain behavior (54).

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REFERENCES

1. Catteall, W. A. (2000) Annu. Rev. Cell Dev. Biol. 16, 521–555
2. Dolphin, A. C. (2006) Br. J. Pharmacol. 147, Suppl. 1, S56–S62
3. Matsuyama, Z., Wakamori, M., Mori, Y., Wakamori, M., Mori, Y., Villaz, M., and De Waard, M. (2002) Eur. J. Neurosci. 16, 883–895
4. Neely, A., Wei, X., Okese, R., Birnbaumer, L., and Stefani, E. (1993) Science 262, 575–578
5. Cornet, V., Bichet, D., Sandoz, G., Marty, I., Brocard, J., Bourin?, E. (2002) J. Biol. Chem. 277, 33598–33603
6. Turner, T. J., Adams, M. E., and Dunlap, K. (1993) Proc. Natl. Acad. Sci. USA 90, 9518–9522
7. Hirning, L. D., Fox, A. P., McColary, E. W., Olivera, B. M., Thayer, S. A., Miller, R. J., and Tsien, R. W. (1988) Science 239, 57–61
8. Bowersox, S. S., Gadoibo, T., Singh, T., Pettus, M., Wang, X. Y., and Luther, R. R. (1996) J. Pharmacol. Exp. Ther. 279, 1243–1249
9. Clasbrummel, B., Osswald, H., and Illes, P. (1999) Br. J. Pharmacol. 96, 101–110
10. Brock, J. A., and Cunnane, T. C. (1999) Br. J. Pharmacol. 126, 11–18
11. Maximov, A., and Bezprozvanny, I. (2002) J. Neurosci. 22, 6939–6952
12. Pragnell, M., De Waard, M., Mori, Y., Tanabe, T., Snutch, T. P., and Campbell, K. P. (1994) Nature 368, 67–70
13. Chen, Y. H., Li, M. H., Zhang, Y., He, L. L., Yamada, Y., Fitzmaurice, A., Shen, Y., Zhang, H., Long, L., and Yang, J. (2004) Nature 429, 675–680
14. Opatowsky, Y., Chen, C. C., Campbell, K. P., and Hirsch, J. A. (2004) Neuron 42, 387–399
15. Van Petegem, F., Clark, K. A., Chatelain, F., and Minor, D. L. J. (2004) Nature 429, 671–675
16. Obermaier, G. J., Schlick, R. Di Basi, M. Subramaniam, P., Gehbart, M., Baumgartner, S., and Flucher, B. E. (2010) J. Biol. Chem. 285, 5776–5791
17. Cormack, B. P., Valdivia, R. H., and Falkow, S. (1996) Gene 173, 33–38
18. Raghib, A., Bertaso, F., Davies, A., Page, K. M., Meir, A., Bogdanov, Y., and Dolphin, A. C. (2001) J. Neurosci. 21, 8495–8504
19. Ferron, L., Davies, A., Page, K. M., Cox, D. J., Leroy, J., Waihte, D., Butcher, A. J., Sellaturay, P., Bolsover, S., Pratt, W. S., Moss, F. J., and Dolphin, A. C. (2008) J. Neurosci. 28, 10604–10617
20. Canti, C., Page, K. M., Stephens, P., and Dolfin, A. C. (1999) J. Neurosci. 19, 6685–6684
21. Canti, C., Davies, A., Berrow, N. S., Butcher, A. J., Page, K. M., and Dolphin, A. C. (2001) J. Biol. Chem. 276, 385–384
22. Page, K. M., Hebbich, F., Margas, W., Pratt, W. S., Nieto-Rostro, M., Chagar, K., Sandhu, K., Davies, A., and Dolphin, A. C. (2010) J. Biol. Chem. 285, 835–844
23. Page, K. M., Hebbich, F., Davies, A., Butcher, A. J., Leroy, J., Bertaso, F., Pratt, W. S., and Dolphin, A. C. (2004) J. Neurosci. 24, 5400–5409
24. Kucharz, K., Krogh, M., Ng, A. N., and Toresson, H. (2009) PLoS ONE 4, e5250
25. Tsubuki, S., Kawasaki, H., Miyashita, N., Inomata, M., and Kawashima, S. (1993) Biochem. Biophys. Res. Commun. 196, 1195–1201
26. Palombella, V. J., Rando, O. J., Goldberg, A. L., and Mantiatis, T. (1996) Cell 84, 773–785
27. Neto, L., Stewart, R. S., Adles, C., Stewart, L. R., Quaglio, E., Biasini, E., Fioriti, L., Chiesa, R., and Harris, E. W. (2003) J. Biol. Chem. 278, 21732–21743
28. Ding, Q., Dimayuga, E., Luo, W., Quaglio, E., Biasini, E., Fioriti, L., Chiesa, R., and Harris, E. W. (2003) J. Biol. Chem. 278, 21732–21743
29. Bourinet, E. (2002) Proc. Natl. Acad. Sci. U.S.A. 99, 1429–1442
30. Flor, H., MacK, T. G., Wagner, D., and Coleman, M. P. (2003) J. Neurosci. Res. 74, 906–916
31. Tran-Van-Minh, A., and Vos, M. C. A. (2010) J. Neurosci. 30, 12856–12867
32. Zhou, R., Patel, S. V., and Snyder, P. M. (2007) J. Biol. Chem. 282, 20207–20212
33. Fotia, A. B., Ekberg, J., Adams, D. J., Cook, D. I., Poronnik, P., and Kumar, S. (2004) J. Biol. Chem. 279, 28930–28935
34. Berrou, L., Klein, H., Bernatchez, G., and Parent, L. (2002) Biophys. J. 83, 1429–1442
35. Richards, M. W., Butler, A. J., and Dolphin, A. C. (2004) TIPS 25, 626–632

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44. Lipscombe, D., Madison, D. V., Poenie, M., Reuter, H., Tsien, R. Y., and Tsien, R. W. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 2398–2402
45. Howe, C. L., Valletta, J. S., Rusnak, A. S., and Mobley, W. C. (2001) Neuron 32, 801–814
46. Altier, C., Garcia-Caballero, A., Simms, B., Walcher, J., Tedford, H., Hermasilla, T., and Zamponi, G. (2009) Soc. Neurosci. Abs. 519.13
47. Campbell, D. S., and Holt, C. E. (2001) Neuron 32, 1013–1026
48. Verma, P., Chierzi, S., Codd, A. M., Campbell, D. S., Meyer, R. L., Holt, C. E., and Fawcett, J. W. (2005) J. Neurosci. 25, 331–342
49. Drinjakovic, J., Jung, H., Campbell, D. S., Strochlic, L., Dwivedy, A., and Holt, C. E. (2010) Neuron 65, 341–357
50. Maltez, J. M., Nunziato, D. A., Kim, J., and Pitt, G. S. (2005) Nat. Struct. Mol. Biol. 12, 372–377
51. Ye, Y., Shibata, Y., Kikkert, M., van Voorden, S., Wiertz, E., and Rapoport, T. A. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 14132–14138
52. Snutch, T. P. (2005) NeuroRx 2, 662–670
53. Bauer, C. S., Nieto-Rostro, M., Rahman, W., Tran-Van-Minh, A., Ferron, L., Douglas, L., Kadurin, I., Sri Ranjan, Y., Fernandez-Alacid, L., Millar, N. S., Dickenson, A. H., Lujan, R., and Dolphin, A. C. (2009) J. Neurosci. 29, 4076–4088
54. Murakami, M., Nakagawasai, O., Yanai, K., Nunoki, K., Tan-No, K., Tadano, T., and Iijima, T. (2007) Brain Res. 1160, 102–112