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Article Addendum

Time course and specificity of the pharmacological disruption of the trafficking of voltage-gated calcium channels by gabapentin

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The mechanism of action of gabapentin is still not well understood. It binds to the $\alpha_2\delta$-1 and $\alpha_2\delta$-2 subunits of voltage-gated calcium channels but has little acute effect on calcium currents in several systems. However, our recent results conclusively demonstrated that gabapentin inhibited calcium currents when applied chronically but not acutely, both in heterologous expression systems and in dorsal root ganglion neurons.[1] In that study we only examined a 40-hour time point of incubation with gabapentin, and here we have extended these results to include the effect of up to 6 and 20 hours incubation with gabapentin on calcium channel currents formed from $Ca_V2.1/\beta_4/\alpha_2\delta$-2 subunits. Gabapentin was significantly effective to inhibit the currents if included for 17–20 hours prior to recording, but it did not produce a significant inhibition if included for 3–6 hours. We previously concluded that gabapentin acts primarily at an intracellular location, requiring uptake into cells. However, this effect is mediated by $\alpha_2\delta$ subunits, being prevented by mutations in either $\alpha_2\delta$-1 or $\alpha_2\delta$-2 that abolish gabapentin binding.[1] Furthermore, we also showed that the trafficking of $\alpha_2\delta$-2 and Ca_V2 channels was disrupted by gabapentin. Here we have also extended that study, to show that the cell-surface expression of $Ca_V2.1$ is not reduced by chronic gabapentin if it is co-expressed with $\alpha_2\delta$-2 containing a point mutation (R282A) that prevents gabapentin binding.

Introduction

Voltage-gated calcium channels (VGCCs) are essential for the function of all excitable cells, and are implicated in many cellular processes.[2,3] They have been divided on the basis of their biophysical properties and pharmacology into L-, N-, P/Q-, R- and T-types.[4] Each calcium channel is composed of a pore-forming $\alpha 1$ subunit, associated with β and $\alpha_2\delta$ accessory subunits (except for T-type channels),[5,6] and for skeletal muscle channels there is also a γ subunit. The α_1 subunit determines the main biophysical properties of the channel, and is modulated by the other subunits.[3,7-10] Mammalian genes encoding ten α_1, four β and four $\alpha_2\delta$ subunits have been identified (reviewed in ref. 11).

The function(s) of the $\alpha_2\delta$ subunit remain poorly investigated; it has a role in trafficking and also influences voltage-dependent and kinetic properties, as investigated by a number of groups.[12-14] The entire α_2 polypeptide is extracellular, whereas δ is predicted to have a transmembrane domain.[12,15,16] We have recently shown that one of the main effects of $\alpha_2\delta$ subunits is to traffic calcium channel heteromers to the plasma membrane, utilising the Von Willebrand factor-A domain which is within α_2.[17]

Of the four cloned $\alpha_2\delta$ subunit genes, $\alpha_2\delta$-1 is the original skeletal muscle subunit, whose distribution is fairly ubiquitous.[5,13,18,19] The $\alpha_2\delta$-2 and $\alpha_2\delta$-3 subunits were subsequently cloned from brain.[13,20] The most recently identified $\alpha_2\delta$-4 is largely non-neuronal.[19] For the Ca_V1 and Ca_V2 class of channels, there does not appear to be a structural specificity concerning which subunits are able to form functional channels together. However, specificity is imposed at the cellular level by selective expression, as certain cell types may contain particular complements of calcium channel subunits, for example cerebellar Purkinje cells primarily express certain splice variants of α_{1A}, both β_{2a} and β_4, and only $\alpha_2\delta$-2.[13]

Experimental nerve crush injury is known to result in an increase in the level of $\alpha_2\delta$-1 mRNA in the damaged sensory neurons (DRGs), particularly the nociceptors, as shown by in situ hybridisation[21] and microarray analysis.[22] There is a corresponding increase in $\alpha_2\delta$-1 protein in DRGs and spinal cord, as determined by Western blot,[23] and by light and electron microscope immunohistochemistry (Bauer et al., in preparation). The mechanism for the elevated expression of $\alpha_2\delta$-1 is unknown, but it may result from enhanced firing of the cells following nerve damage.

Gabapentin and its analogue pregabalin are of therapeutic benefit in the alleviation of neuropathic hyperalgesia and allodynia that occurs following nerve damage (reviewed in ref. 24). They also reduce hyperalgesia and allodynia in experimental models of neuropathic pain, but have no effect on control responses.[25,26] Although gabapentin was

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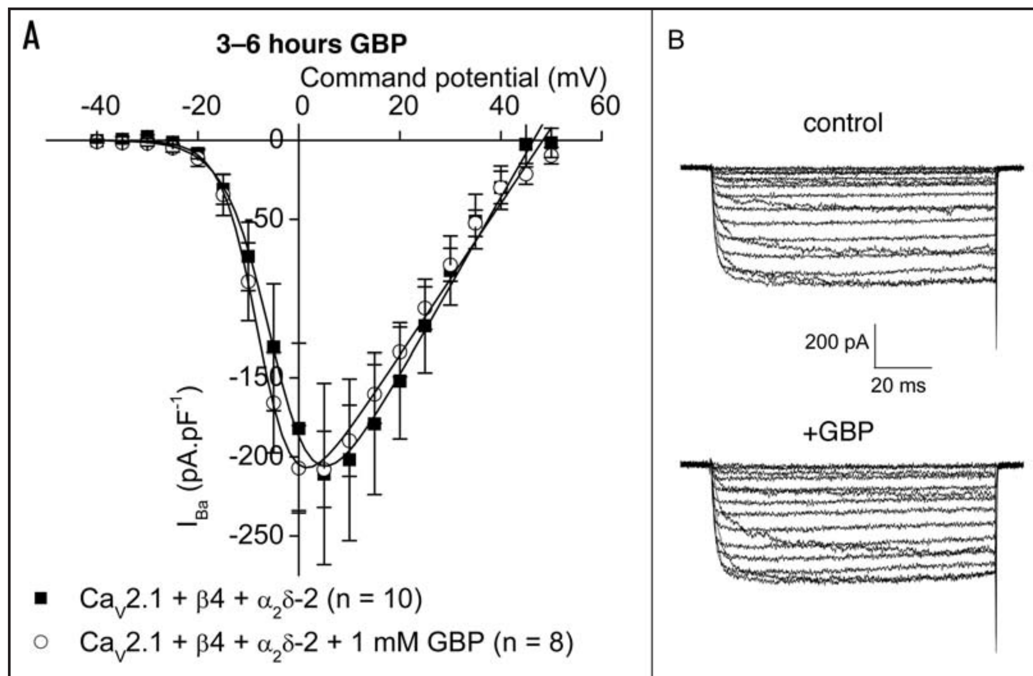

Figure 1. Gabapentin does not inhibit I_{Ba} following heterologous expression, when applied for 3–6 hours. (A) Current density-voltage (IV) relationships for $Ca_V2.1/\beta_4/\alpha_2\delta$-2 currents in the absence or presence of gabapentin (GBP, 1 mM, ○, n = 8) for 3–6 hours (or H_2O as control, ■, n = 10), immediately prior to recording. Gabapentin was also present in the medium following re-plating of the cells, and in the medium during recording. There was no reduction in I_{Ba} at any potential. (B) Examples of currents resulting from step potentials from -90 mV to between -15 and +35 mV in 5-mV increments, under control conditions and in the presence of 1 mM gabapentin. Calibration bars refer to both sets of traces.

originally developed as a rigid analogue of GABA, it has no activity at GABA-A or GABA-B receptors or GABA transporters (reviewed in ref. 27). Gabapentin was then identified to bind to the calcium channel $\alpha_2\delta$-1 and -2 subunits,[28,29] although it produces little or no acute block of calcium currents (reviewed in ref. 10).

Conclusive evidence has recently been obtained that the efficacy of gabapentin and pregabalin in the alleviation of neuropathic pain occurs via binding to $\alpha_2\delta$-1.[30] The authors made a knock-in mouse strain with a mutation in $\alpha_2\delta$-1 (R217A) that renders the mice insensitive to gabapentin. With the knowledge that the main effect of $\alpha_2\delta$ subunits is on calcium channel trafficking,[17] we have recently shown that chronically applied, but not acute, gabapentin impedes calcium channel trafficking to the cell surface.[1] Our results indicated that gabapentin acts primarily at an intracellular site to impede trafficking. This requires both uptake into the cell and binding to $\alpha_2\delta$ subunits.

In the present study we have extended these results to provide a time course for the effect of gabapentin, and to show that the effect of gabapentin on trafficking $\alpha_2\delta$-2 subunits and the associated α1 subunits to the plasma membrane is lost when a mutation is made in the $\alpha_2\delta$ subunit, such that it does not bind gabapentin.

Results

Time course of the effect of chronic gabapentin to inhibit calcium currents. The tsA 201 cells were transfected with the cDNA combination $Ca_V2.1/\alpha_2\delta$-2/β4, and recordings were made 40–48 hours after transfection. In previous experiments,[1] gabapentin was included in the medium for the entire period between transfection and re-plating for electrophysiological recording, whereas here it was included for the periods stated in the legends to Figures 1 and 2. We previously showed that gabapentin inhibited calcium currents when applied chronically for ~40 hours, but not when applied acutely for 10 min.[1] We now show that there was no effect of 1 mM gabapentin when it was applied for 3–6 hours prior to recording (Fig. 1A and B), but there was a significant inhibition by gabapentin when it was applied for 17–20 hours prior to recording (Fig. 2A and B). The time course of the effect of gabapentin (Fig. 2C) includes the mean data for 10 minutes and 40 hours, obtained previously, under conditions described in ref. 1, for completeness .

Lack of effect of gabapentin on R282A-$\alpha_2\delta$-2. We previously showed that chronically applied gabapentin reduced the cell surface expression of $Ca_V2.1$ and $\alpha_2\delta$-2 in transfected cells, when applied chronically for the entire period between transfection and fixation of the cells (72 hours). This correlated with our electrophysiological findings.[1] In that study, we also showed that the effect of chronic gabapentin on calcium channel currents was lost when the $\alpha_2\delta$-2 subunit was mutated R282A $\alpha_2\delta$-2 such that it did not bind gabapentin.[1] We now show corresponding evidence for the effect on cell surface expression, providing confirmation that the effect of gabapentin on trafficking is via the $\alpha_2\delta$ subunit. In Figure 3A (left) the effect is shown of chronic gabapentin application to reduce cell surface expression of $Ca_V2.1$-2HA (green) and $\alpha_2\delta$-2 (red), as described previously.[1] In contrast no effect of gabapentin was observed when the R282A mutant form of $\alpha_2\delta$-2 was used (Fig. 3B, left). In permeabilized cells gabapentin increased the intracellular retention of wild type $\alpha_2\delta$-2 and $Ca_V2.1$-2HA (Fig. 3A, right), as previously described.[1] In contrast there was more intracellular retention of both R282A $\alpha_2\delta$-2 and $Ca_V2.1$-2HA when they were co-expressed in the absence of gabapentin (Fig. 3B, right) and the addition of gabapentin had no additional effect (Fig. 3B, right).

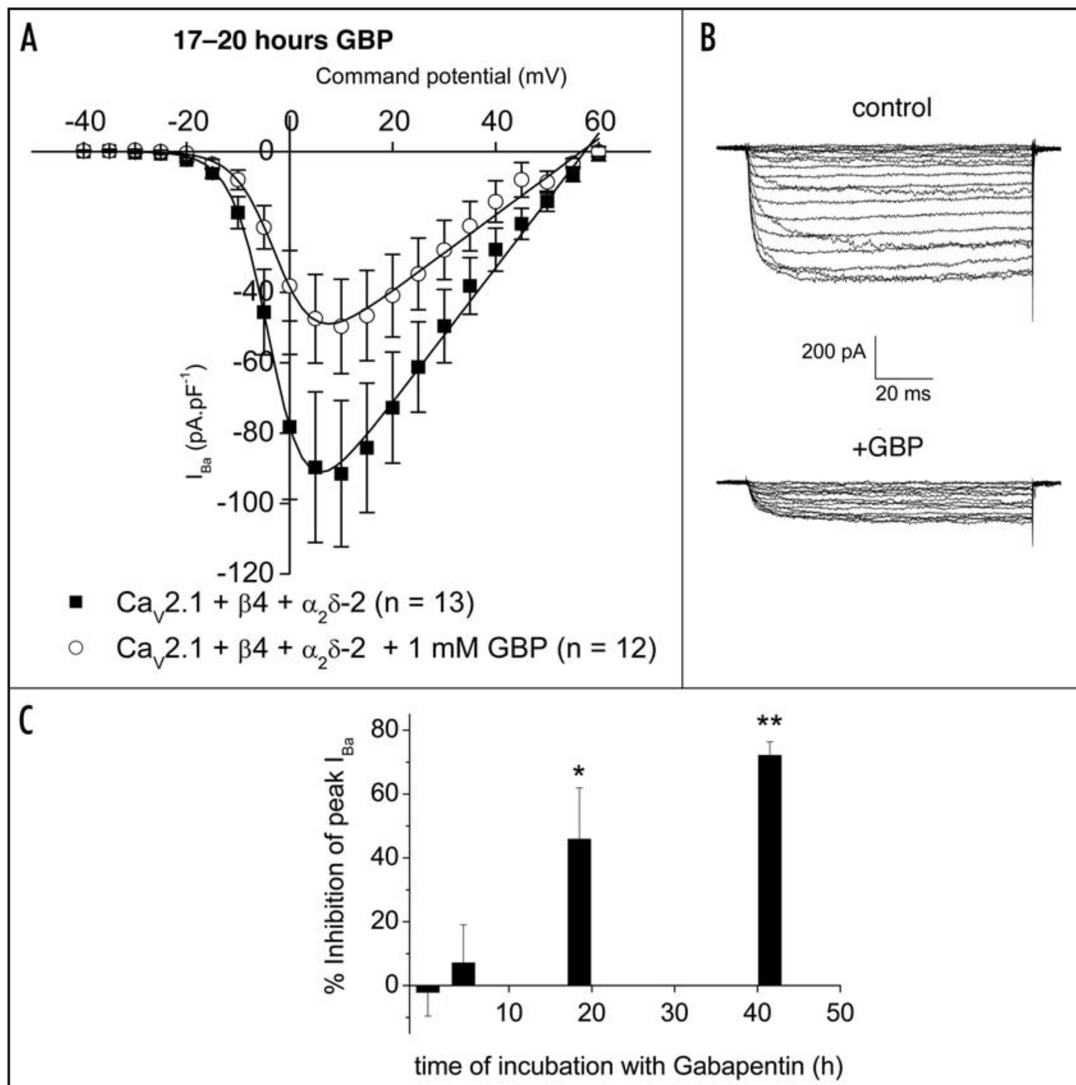

Figure 2. Gabapentin inhibits I_{Ba} following heterologous expression, when applied for 17–20 hours. (A) Current density-voltage (IV) relationships for $Ca_V2.1/\beta_4/\alpha_2\delta$-2 currents in the absence or presence of gabapentin (GBP, 1 mM, ○, n = 12) for 17–20 hours (or H_2O as control, ■, n = 13), immediately prior to recording. Gabapentin was also present in the medium following re-plating of the cells, and in the medium during recording. (B) Examples of currents resulting from step potentials from -90 mV to between -20 and +55 mV in 5 mV increments, under control conditions and in the presence of 1 mM gabapentin. Calibration bars refer to both sets of traces. (C) The bar chart shows time course of the inhibition by 1 mM gabapentin for exposures from 10 min. (n = 5) and ~40 hours (n = 11.) There was a significant reduction in peak I_{Ba} at +10 mV at both 17–20 hours (*p = 0.012) and ~40 hours (**p = 0.0013).[1] The 10 min. and 40 hours data were obtained previously,[1] using conditions described therein.

Discussion

The mechanism of action of gabapentin has been under debate for many years. It was identified in 1996 that the gabapentin binding site was the calcium channel auxiliary $\alpha_2\delta$-1 subunit,[28] and it was therefore assumed that gabapentin would have its action via inhibition of calcium currents. Although it was initially reported that there was an acute inhibition of calcium currents by about 20% in cultured DRGs, depending on culture conditions,[36] several studies have reported that there is little or no acute inhibition of calcium currents either in neurons or in heterologous expression systems.[1,10,26] However, in DRGs from $\alpha_2\delta$-1 overexpressing mice, an inhibition of calcium currents by acute gabapentin was reported, over a period of 5–10 minutes.[26]

The literature is similarly mixed regarding inhibition of transmitter release. Gabapentin and pregabalin were reported to produce a small (~10%) inhibition of release from both glutamate and GABA synapses in hippocampal cultures, and also to reduce osmotically induced release, although this is known to be a calcium-independent process.[37] Furthermore, gabapentin inhibited the facilitation of K^+-stimulated release by a PKC activator, but not basal K^+-stimulated release of glutamate from trigeminal slices.[38]

In experiments on synaptic transmission, gabapentin and pregabalin were reported to reduce the frequency of miniature and evoked synaptic currents.[39] Also, it was found that gabapentin inhibited both glutamatergic and glycinergic synaptic transmission in the mouse spinal cord dorsal horn through a preferential block of P/Q-type Ca^{2+} channels,[40] but it was also reported that gabapentin

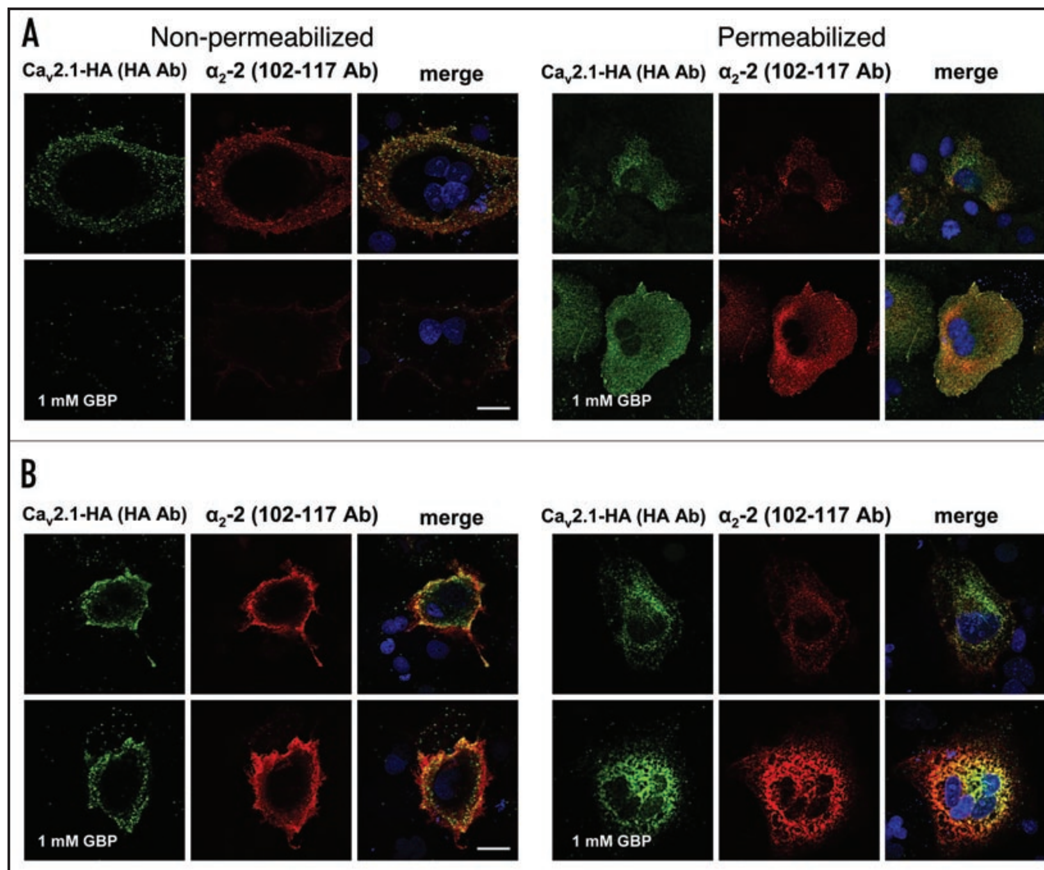

Figure 3. Effect of chronic GBP on the plasma membrane localization of $Ca_V2.1$ and $\alpha_2\delta$-2 or R282A $\alpha_2\delta$-2 in COS-7 cells. (A) $Ca_V2.1$-2HA was co-transfected with β_4 and $\alpha_2\delta$-2 and cultured for 72 hours either in the absence or in the presence of GBP (1 mM). Cells were then fixed and either not permeabilized (left) or permeabilized (right) before immunocytochemical localization of $Ca_V2.1$ (HA Ab, left) and $\alpha_2\delta$-2 [$\alpha_2\delta$-2 (102–117) Ab, center]. Immunostaining was visualized using a magnification x 63 objective. Merged images are shown (right) ($Ca_V2.1$ is shown in green and $\alpha_2\delta$-2 in red, with regions of co-localization in orange-yellow). Nuclear staining (blue, DAPI) is shown in the merged images. Images show 1 μm optical sections of data representative of three independent experiments. (Scale bar: 30 μm). No signal was observed in non-transfected cells or in the absence of primary Abs. (B) Images are obtained as in (A), but the cells were co-transfected with $Ca_V2.1$-2HA, β_4 and $\alpha_2\delta$-2 R282A. (Scale bar: 30 μm).

did not alter P/Q-type Ca^{2+} channel-mediated synaptic transmission in the hippocampus in vitro.[41] A selective effect on the inhibition of P/Q rather than N-type channels would be difficult to explain, as we have not found any selectivity in the association of $\alpha_2\delta$ subunits with $Ca_V2.1$, rather than $Ca_V2.2$ channels.

Our previous results showed conclusively that gabapentin inhibits calcium currents when applied chronically, by an intracellular effect on calcium channel trafficking.[1] In that paper, the only time point measured was after 40 hours of incubation with gabapentin, therefore it was present from immediately after transfection. Here we show that gabapentin also significantly inhibited calcium channel currents by 45% after 17–20 hours of incubation, but there was no significant inhibition after 3–6 hours.

Gabapentin was used in this study at a concentration of 1 mM, because it was added to the culture medium, which contains high concentrations of large neutral amino-acids.[42,43] For example, both isoleucine and leucine are present at 800 μM, and valine at 400 μM in the culture medium used. These amino acids compete with gabapentin for uptake into cells via system L transporters,[42] and also compete with gabapentin for binding to $\alpha_2\delta$-1.[43]

We also show that the gabapentin-mediated reduction of cell-surface expression of $\alpha_2\delta$-2 and $Ca_V2.1$ was prevented by substitution of the gabapentin-insensitive $\alpha_2\delta$-2 mutant (R282A $\alpha_2\delta$-2). This indicates that gabapentin is acting indirectly on $Ca_V2.1$, via binding to $\alpha_2\delta$-2, either to reduce its trafficking to the plasma membrane, or to affect its endocytosis.

It is still unclear what the normal function of the gabapentin binding site on $\alpha_2\delta$ subunits might be, and whether it is occupied by an unknown endogenous ligand. In relation to this, we have identified that $\alpha_2\delta$ subunits are concentrated in cholesterol-rich microdomain (lipid raft) fractions both in neuronal tissue and in transfected cells.[44] The apparent affinity of $\alpha_2\delta$-2 for gabapentin is increased markedly in the cholesterol-rich microdomain fractions.[44] The likely explanation for this is that there is an endogenous molecule that binds to $\alpha_2\delta$, and dissociates during the purification process.[44] This molecule would interact either with the same site as gabapentin, or would allosterically modulate that site. It is possible that this unknown endogenous molecule is a neutral ligand or might be a positive modulator of $\alpha_2\delta$ subunit function. In regard to this we have shown that R217A $\alpha_2\delta$-1 and the equivalent mutation in $\alpha_2\delta$-2 (R282A) have reduced functionality, again suggesting the possibility that the gabapentin binding site might normally be occupied by an endogenous ligand, whose presence might be required for the full functionality of $\alpha_2\delta$.[30,44]

Methods

Construction and heterologous expression of cDNAs. Mammalian cell lines were transfected with cDNAs for $Ca_V2.1$ or $Ca_V2.1$-2HA[1] in conjunction with rat β_4 and mouse $\alpha_2\delta$-2 (AF247139).[31] The cDNAs were subcloned into the pMT2 vector for expression in tsA-201 or Cos-7 cells. The cDNA for green fluorescent protein (mut3 GFP)[32] was included in the transfection to identify transfected cells from which electrophysiological recordings were made. Transfection was performed as described previously.[33] Cells were re-plated at least 2 hours prior to electrophysiological recording.

Electrophysiology. Calcium channel expression in tsA-201 cells was investigated by whole cell patch clamp recording, essentially as described previously.[34] The internal (pipette) and external solutions and recording techniques were similar to those previously described.[35] The patch pipette solution contained in mM: Cs-aspartate, 140; EGTA, 5; $MgCl_2$, 2; $CaCl_2$, 0.1; K_2ATP, 2; Hepes, 10; pH 7.2, 310 mOsm with sucrose. The external solution contained in mM: tetraethylammonium (TEA) Br, 160; KCl, 3; $NaHCO_3$, 1.0; $MgCl_2$, 1.0; Hepes, 10; glucose, 4; $BaCl_2$, 5, pH 7.4, adjusted to 320 mosM with sucrose.

Immunocytochemistry and imaging. Cos-7 cells were transfected with cDNAs for human $Ca_V2.1$-2HA in the pRK5 vector, rat β_4 and either WT or R282A mouse $\alpha_2\delta$-2, both in the vector pMT2, in a 3:2:2 ratio. The HA-tagged $Ca_V2.1$ construct ($Ca_V2.1$-2HA) was made from human $Ca_V2.1$ (AF 004883) with a double-HA tag in an extracellular loop in Domain IV. Immunolabeling was performed 72 hours after transfection; the cells were washed twice in Tris-buffered saline (TBS) and fixed with 4% paraformaldehyde in TBS for 5 minutes, then primary Abs were applied overnight at 4°C. For labelling all epitopes, cells were permeabilized by incubating twice for 7 minutes in a 0.02% solution of Triton X-100 in TBS, whereas, for labelling only the extracellular epitopes of the channels inserted in the plasma membrane, cells were not permeabilised. Cells were washed twice with TBS for the same time period. The primary anti-$\alpha_2\delta$-2 Ab (102–117) was used at 1–2 $\mu g.ml^{-1}$, followed by the secondary FITC-conjugated goat anti-rabbit Ab (Sigma, Poole, UK; 1:500). A rat monoclonal anti-HA Ab (Roche) was used at 0.2 $\mu g.ml^{-1}$, with a biotinylated anti-rat IgG (Sigma, 0.6 $\mu g.ml^{-1}$), followed by streptavidin-Texas Red (Molecular Probes, Eugene, Oregon, 2 $\mu g.ml^{-1}$). In some experiments, the nuclear dye 4',6-diamidino-2-phenylindole (DAPI, 500 nM, Molecular Probes) was also used to visualize the nucleus. Cells were mounted in Vectashield (Vector laboratories, Burlingame, CA) to reduce photobleaching, and examined on a confocal laser scanning microscope (Zeiss LSM), using a x 63 (1.4 NA) oil-immersion objective. Optical sections were 1 μm. Photomultiplier settings were kept constant in each experiment and all images were scanned sequentially. Data illustrated are representative of more than ten cells from at least three independent experiments.

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