Constitutive activity of the Ghrelin receptor reduces surface expression of voltage-gated Ca\(^{2+}\) channels in a Ca\(_{\alpha\beta}\)-dependent manner

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INTRODUCTION
Voltage-gated Ca\(^{2+}\) (Ca\(_{\alpha}\)) channels are instrumental in coupling a change in transmembrane voltage to Ca\(^{2+}\) influx that, in turn, regulates numerous critical neuronal functions. Depending on cell type, developmental stage and subcellular location, different Ca\(_{\alpha}\) subtypes are involved in orchestrating Ca\(^{2+}\)-dependent signaling; Ca\(_{\alpha}1\) and Ca\(_{\alpha}2\) channels trigger transcription-dependent forms of synaptic plasticity (Ca\(_{\alpha}1.2\) and Ca\(_{\alpha}1.3\)) (Wheeler et al., 2008) and fast neurotransmitter release (Ca\(_{\alpha}2.1-3\)) (Dunlap et al., 1995; Catterall and Few, 2008; Pan and Zucker, 2009), whereas, Ca\(_{\alpha}3\) and Ca\(_{\alpha}1.3\) channels, that activate closer to the resting membrane potential, are involved in regulating cell excitability and shaping neuronal firing patterns (Molineux et al., 2006; Perez-Reyes, 2003; McKay et al., 2006; Xu and Lipscombe, 2001).

The temporal features and spatial distribution of each Ca\(_{\alpha}\) channel subtype are strongly related to the specific roles of Ca\(_{\alpha}\) channels in different cells (Dolphin, 2012). Ca\(_{\alpha}\) channels associate with various proteins and these auxiliary subunits influence their trafficking to the plasma membrane (Dolphin, 2016; Felix et al., 2013; Simms and Zamponi, 2012). Ca\(_{\alpha}\beta\) and Ca\(_{\alpha}\delta\beta\) are important auxiliary subunits that promote displacement of Ca\(_{\alpha}\alpha\beta\) subunits from the endoplasmic reticulum (ER), and influence forward trafficking as well as stability at the plasma membrane (Felix et al., 2013; Dolphin, 2012). The influence of auxiliary subunits on Ca\(_{\alpha}\) channel densities is less clear (Bichet et al., 2000; Fang and Colecraft, 2011; De Waard et al., 1994). Post-translational modifications, including asparagine-linked glycosylation, are known to promote cell surface expression of Ca\(_{\alpha}3.2\) (Weiss et al., 2013; Orestes et al., 2013) and indicate differences between the basic mechanisms controlling the plasma membrane density of different Ca\(_{\alpha}\) channel subtypes.

An understanding of the mechanism that control Ca\(_{\alpha}\) channel surface density in neurons is essential for a complete view of how cellular Ca\(^{2+}\) signals are regulated. Studies that explore Ca\(_{\alpha}\) channel trafficking have started to contribute to knowledge in this field but there is much that we still do not know (Marangoudakis et al., 2012; Erickson et al., 2007). Activated GPCRs can promote Ca\(_{\alpha}\) channel removal from the plasma membrane by internalization (Simms and Zamponi, 2012) and, in some cases, GPCR and Ca\(_{\alpha}\) channels are internalized together (Kislevsky and Zamponi, 2008). Our group has shown that constitutively active growth hormone secretagogue receptor type 1a (GHSR), reduces the density of Ca\(_{\alpha}2.1\) and Ca\(_{\alpha}2.2\) channel currents in both a heterologous expression system and in hypothalamic neurons (Lopez Soto et al., 2015). Here, we show that constitutively active GHSR regulates surface expression of several Ca\(_{\alpha}\) channel subtypes. We present evidence that GHSR-mediated reduction in the current of Ca\(_{\alpha}\) channels depends on the presence of an auxiliary Ca\(_{\alpha}\beta\) subunit, and functions by downregulating forward trafficking of Ca\(_{\alpha}\) channels to the plasma membrane. Our data reveal a new link between Ca\(_{\alpha}\beta\) subunits and GHSR-dependent control of Ca\(_{\alpha}\) channel activity.

RESULTS
We have shown previously that constitutively active GHSR targets presynaptic Ca\(_{\alpha}2.1\) and Ca\(_{\alpha}2.2\) in hypothalamic neurons and reduces their surface density (Lopez Soto et al., 2015). Here, we investigated whether constitutively active GHSR influences surface densities of other subtypes of Ca\(_{\alpha}\) channels. We first assessed whether GHSR influences the size of Ca\(_{\alpha}1.2\) and Ca\(_{\alpha}1.3\) channel current densities. We used whole-cell patch clamp recording with 2 mM Ca\(^{2+}\) as the charge carrier. We recorded Ca\(_{\alpha}\) channel currents from tsA201 cells co-transfected with Ca\(_{\alpha}1.2\) or Ca\(_{\alpha}1.3\), together with auxiliary subunits Ca\(_{\alpha}\alpha\delta\beta\) and Ca\(_{\alpha}\beta\delta\), either with GHSR or an empty plasmid. Compared to control cells, we found only very small Ca\(_{\alpha}1.2\) and Ca\(_{\alpha}1.3\) currents in cells that express GHSR (Fig. 1A),
but this effect of GHSR was blocked by pre-incubation with the inverse GHSR agonist SPA (Fig. 1A). This suggests that the inhibitory actions of GHSR in tsA201 cells depend on constitutive activity of GHSR. Others have shown that GHSR is expressed at relatively high levels in hypothalamic neurons (Zigman et al., 2006). Furthermore, we have shown that constitutively active GHSR inhibits CaV1.2 and CaV1.3 currents in hypothalamic neurons (Lopez Soto et al., 2015). We, therefore, tested if GHSR also inhibits endogenous CaV1 channels in hypothalamic neurons by comparing CaV1 currents in hypothalamic neuronal cultures derived from wild type and GHSR-deficient (GHSR-null) mice. Total CaV currents in neurons isolated from GHSR-null mice were higher than those from wild type (Lopez Soto et al., 2015). To assess the relative size of CaV1 currents in hypothalamic neurons, in the presence and absence of GHSR, we used the dihydropyridine agonist Bay K 8644. The use of Bay K 8644 to induce increase in CaV currents is a robust method to augment the density of CaV1 channels. Bay K 8644 (BayK) effect (5 µM) on the Ba2+ current from control cells (Fig. 4C, top and bottom panels, respectively). We also coexpressed GHSR with CaV1.2, CaV1.3, CaV2.1, CaV2.2, CaV2.3, and GHSR (+GHSR, n=13) pre-incubated or not with SPA 1 µM (+SPA, n=9) and from controls transfected with empty plasmid (-GHSR, n=19), and average I Ca for each condition (left). Representative CaV current traces from tsA201 cells co-transfected with CaV1.2, CaV α2δ2, CaV β 3 and GHSR (+GHSR, n=8) or from controls transfected with empty plasmid (-GHSR, n=12), and average I Ca for each condition (left). Representative CaV current traces from tsA201 cells co-transfected with CaV1.3, CaV α2δ2, CaV β 3 and GHSR (+GHSR, n=13) pre-incubated or not with SPA 1 µM (+SPA, n=9) and from controls transfected with empty plasmid (-GHSR, n=19), and average I Ca for each condition (right). (B) Representative traces of the Bay K 8644 (BayK) effect (5 µM) on the Ba2+ current from GHSR-deficient (GHSR-null, n=5) and wild-type (Wild type, n=7) hypothalamic neurons (left), and average I Ca increase (right). Error bars represent mean±s.e.m., individual points represents current registered (A) or current increase for each cell (B). Kruskal–Wallis with Dunn’s post-test (CaV1.3), Mann–Whitney test (CaV1.2) (A), and Student’s t-test (B).

Fig. 1. GHSR constitutive activity reduces CaV1.2 and CaV1.3 currents in tsA201 cells, and reduces native CaV1 currents in cultured hypothalamic neurons. (A) Representative CaV current traces from tsA201 cells co-transfected with CaV1.2, CaV α2δ2, CaV β 3 and GHSR (+GHSR, n=8) or from controls transfected with empty plasmid (-GHSR, n=12), and average I Ca for each condition (left). Representative CaV current traces from tsA201 cells co-transfected with CaV1.3, CaV α2δ2, CaV β 3 and GHSR (+GHSR, n=13) pre-incubated or not with SPA 1 µM (+SPA, n=9) and from controls transfected with empty plasmid (-GHSR, n=19), and average I Ca for each condition (right). (B) Representative traces of the Bay K 8644 (BayK) effect (5 µM) on the Ba2+ current from GHSR-deficient (GHSR-null, n=5) and wild-type (Wild type, n=7) hypothalamic neurons (left), and average I Ca increase (right). Error bars represent mean±s.e.m., individual points represents current registered (A) or current increase for each cell (B).
membrane CaV2.2-GFP signal in cells not expressing CaVα2δ (Fig. 4C, middle panel) and this signal was reduced by GHSR coexpression by an amount that was similar to the reduction observed in cells expressing GHSR, CaV2.2, CaVβ and CaVα2δ (Fig. 4C, bottom panel). Our results suggest that CaVβ, but not CaVα2δ, is required for the inhibitory actions of constitutively active GHSR on CaV channels, and that the actions of GHSR involve reduced expression of CaV2.2 at the plasma membrane.

The effects of GHSR on CaV current density might involve impaired forward trafficking from the ER and Golgi and/or enhanced internalization from the plasma membrane to recycling endosomes (RE) (Simms and Zamponi, 2012). To distinguish

Fig. 2. GHSR constitutive activity fails to reduce CaV3.2 density on the plasma membrane. (A) Representative CaV current traces (left) from tsA201 cells co-transfected with CaV3.2 and GHSR (+GHSR, n=10) or empty plasmid (-GHSR, n=15), and average I_{Ca} for each condition (right). (B) Photomicrographs (left) and average of GFP plasma membrane signal (in percent) (right) of tsA201 cells co-transfected with CaV3.2-GFP and GHSR (+GHSR, n=88) and from controls transfected with empty plasmid (-GHSR, n=94). Green and red signals correspond to the eGFP tag on CaV3.2 and the CellMask membrane marker, respectively. Scale bar: 10 μm. Error bars represent mean±s.e.m., individual points represent each cell analyzed.

Fig. 3. GHSR constitutive activity reduces CaV3.2 density on plasma membrane and, consequently, the CaV3.2 current in a CaVβ3-dependent manner. (A) Representative CaV current traces (left) from tsA201 cells co-transfected with CaV3.2, CaVβ3 and GHSR (+GHSR) pre-incubated or not with SPA 1 μM (+GHSR+SPA), and from controls transfected with empty plasmid (-GHSR) and average I_{Ca} in presence of increasing CaVβ3/CaV3.2 molar ratios (right). (B) Photomicrographs (left) and average of GFP plasma membrane signal (in percent) (right) of tsA201 cells co-transfected with CaV3.2-GFP, CaVβ3 and GHSR (+GHSR, n=21) pre-incubated or not with SPA 1 μM (+GHSR+SPA, n=14) and from controls transfected with empty plasmid (-GHSR, n=27). Green and red signals correspond to the eGFP tag on CaV3.2 and the membrane marker CellMask, respectively. Scale bar: 10 μm. Dots represent mean±s.e.m., numbers in brackets represent the number of analyzed cells in A. Error bars represent mean±s.e.m. and individual points represent each cell analyzed in B. Mann–Whitney test and Kruskal–Wallis with Dunn’s post-test (A). Kruskal–Wallis with Dunn’s post hoc test (B).
between these possibilities, we analyzed the subcellular localization of Ca\textsubscript{V} channels in the presence or absence of GHSR. We used eGFP-tagged Ca\textsubscript{V}\textsubscript{2.1} and Ca\textsubscript{V}\textsubscript{2.2} (Ca\textsubscript{V}\textsubscript{2.1}-eGFP and Ca\textsubscript{V}\textsubscript{2.2}-eGFP, respectively), as well as genetically encoded plasma membrane, ER, Golgi and RE markers to study Ca\textsubscript{V} channels distribution among these different compartments in tsA201 cells. We know that the inhibitory effects of GHSR on Ca\textsubscript{V}2.2 are observed in cells that express either the Ca\textsubscript{V}\textsubscript{2.1} or the Ca\textsubscript{V}\textsubscript{2.2} subtype (Lopez Soto et al., 2015). Ca\textsubscript{V}\textsubscript{2.2} can be palmitoylated and this modification increases its interaction with the plasma membrane (Chien et al., 1995; Chien et al., 1996; Chien et al., 1998), whereas Ca\textsubscript{V}\textsubscript{2.1} is a soluble protein and only migrates to the plasma membrane when in complex with Ca\textsubscript{V}\textsubscript{2.1} (Bichet et al., 2000). Consistent with our functional studies, we observed a reduced Ca\textsubscript{V}\textsubscript{2.1}-eGFP signal at the plasma membrane when GHSR is coexpressed and this effect was blocked by SPA pre-incubation (Fig. 5A). We also found a concomitant increase in the Ca\textsubscript{V}\textsubscript{2.2}-eGFP signal at the ER, accompanied by a mild decrease in the proportional amount of Ca\textsubscript{V}\textsubscript{2.2}-eGFP located at Golgi complex. Under these conditions, the Ca\textsubscript{V}\textsubscript{2.2}-eGFP signal in recycling endosomes was unchanged across different experimental conditions (Fig. 5B). We also assayed changes in the distribution Ca\textsubscript{V}\textsubscript{2.1}-eGFP that were due to the presence of GHSR and found they were similar to the distribution of Ca\textsubscript{V}\textsubscript{2.2}-eGFP (Fig. 6). In agreement with this result, we found that dominant-negative version of Rab11b, a protein that controls internalization of Ca\textsubscript{V}\textsubscript{2.1} to endosomes, does not alter the reduction of the Ca\textsubscript{V} current density caused by GHSR [Ca\textsubscript{V}2.2+Ca\textsubscript{V}\textsubscript{2.1} and GHSR (+GHSR, n=29) or from controls transfected with empty plasmid (-GHSR, n=27) (bottom)]. Green and red signals correspond to the eGFP tag on Ca\textsubscript{V}2.2 and the membrane marker CellMask, respectively. Scale bars: 10 \mu m. Error bars represent mean±s.e.m. and individual points represent each cell analyzed. Mann–Whitney test.

Next we asked if GHSR can modify the subcellular location of Ca\textsubscript{V}\textsubscript{2.1} and Ca\textsubscript{V}\textsubscript{2.2} subunits independently of the Ca\textsubscript{V} channel, by repeating the above experimental series in the absence of Ca\textsubscript{V}\textsubscript{2.1}. Under these conditions, without Ca\textsubscript{V}2.2, GHSR coexpression failed to modify the distribution of Ca\textsubscript{V}\textsubscript{2.1} and Ca\textsubscript{V}\textsubscript{2.2}. As reported by others (Bichet et al., 2000), Ca\textsubscript{V}\textsubscript{2.1} alone does not traffic to the plasma membrane (Fig. 7). We also tested if the Ca\textsubscript{V}\textsubscript{2.1} and Ca\textsubscript{V}\textsubscript{2.2} interaction is required for the inhibitory effect of constitutive active GHSR. We assayed the Trp391Ala mutant of Ca\textsubscript{V}2.2 mutant (Ca\textsubscript{V}2.2W391A) that has
impaired affinity for CaVβ (Van Petegem et al., 2008; Leroy et al., 2005). As shown in Fig. 8A, this mutant failed to block the GHSR inhibitory effect when CaVβ3 or CaVβ2a were present. Moreover, we also assayed a C-terminally truncated form of CaVβ2a (CaVβ2aTF8n) that has been reported to fail to increase CaV2.1 currents and to change activation parameters (Leyris et al., 2009), suggesting it is unable to interact with the CaVα1 subunit. As we show in Fig. 8B, GHSR impairs the CaV2.2 current in the presence of CaVβ2aTF8n.

Taking together, these experiments indicate that a Trp391Ala change in CaVα1 or truncation of CaVβ are not sufficient to block the inhibitory effect of GHSR.

Our data indicate that constitutively active GHSR interferes with the surface expression of CaV1, CaV2 and CaV3 channels by promoting the retention of CaVα1 subunits in the ER when CaVβ subunits are present.

**DISCUSSION**

GHSR is crucially important in the regulation of appetite and bodyweight, and in learning and memory. Here, we extend previous studies of the effect constitutively active GHSR exerts on CaV2 channels, and show that GHSR also downregulates the activity of CaV1 and CaV3 channels. GHSR prevents CaV channels from leaving the ER, but only when CaVβ subunits are present, resulting in reduced density of surface channel.

GPCR-dependent regulation of neuronal CaV channels after receptor and channel trafficking in many cases, including that of the mu 1 opioid receptor (OPRM1), opioid-receptor receptor-like 1 (ORL1, also known as OPRL1), dopamine receptors (DRDs) and γ-aminobutyric acid (GABA) type B receptor subunits (GABBR1 and GABBR2). This form of modulation is long lasting and independent of neuronal activity (Gray et al., 2007). Several of these receptors reduce the surface density of CaV channels by internalizing the channel or co-internalizing a GPCR–channel complex. ORL1 reduces CaV channel density at the plasma membrane after long exposures to the agonist, and this process implicates increasing trafficking to early endosomes or to lysosomes (Altier et al., 2006). Removal of channels from the surface is unlikely to participate in the constitutively active GHSR mechanism since we failed to observe any increase of channel complexes on the recycling endosome. However, as we report for GHSR, other GPCRs can regulate CaV channel activity and surface density in the absence of agonist. For instance, ORL1 exerts a negative modulation of CaV2.2 channel activity in absence of nociceptin, by forming a complex between itself and the CaV2.2 channel (Beedle et al., 2004).
Moreover, DRD1 and DRD2 interact directly with CaV2.2 to increase channel surface expression levels under basal conditions, whereas agonist-activated DRD1 and DRD2 internalize together with CaV channels (Kisilevsky and Zamponi, 2008; Kisilevsky et al., 2008). These reports have shown that GPCRs are able to impact on channel trafficking by directly interacting with the channel itself. In the case of GHSR, we know that the effect depends on the degree of GHSR gene expression (Lopez Soto et al., 2015), which opens the possibility that chronic inhibition of CaV trafficking needs direct interaction of GPCR and the CaV channel.

We have shown here that constitutive active GHSR reduces forward trafficking of CaV channels only when the CaVβ subunit is present. Indeed, the ratio of CaVβ to CaVα1 influences how much CaV3 currents are downregulated by GHSR. As this effect depends on the stoichiometry of channel subunits, we suggest that the interaction of channels subunits is needed such that GHSR is able to exert its inhibitory effect. Consistent with this, we found that CaVα1 must be present for GHSR to modify the subcellular localization of eGFP-tagged CaVβ. However, we also found that the inhibitory effect of GHSR was not changed by CaV2.2W391A or CaVβ2aTF8n. These results suggest that the interaction between CaVα1 and CaVβ, mediated by W391 in the α-interaction domain (AID) and/or the segment lacking in CaVβ2aTF8n, is not required for the GHSR inhibitory effect. More experiments are, therefore, required to conclude whether the presence of CaVβ alone is sufficient to mediate the inhibitory effect of GHSR.

Considering that the chronic inhibition of CaV channels by GHSR relies on retention of CaV channels at the ER — during which we observed a mild decrease in the proportional amount of channels located at Golgi complex (CaVβ2a only) — we postulate that CaVβ acts as an inhibitor for forward trafficking when GHSR is active in addition to its established stimulatory role. In this regard, previous studies have shown that CaVβ controls forward trafficking of CaV channels (Simms and Zamponi, 2012) by preventing channel ubiquitylation and posterior degradation through the proteasome, by masking a putative ER-retention domain (Altier et al., 2011; Fang and Colecraft, 2011). However, several reports suggest a dual function of CaVβ, as (1) stimulator of forward trafficking and (2) mediator of trafficking to endosomes. Hidalgo’s group has postulated a mechanism in which small GTPases and dynamin simultaneously interact with CaVβ dimers and, as a consequence, stimulate the endocytosis of channel complexes (Gonzalez-Gutierrez et al., 2007; Miranda-Laferte et al., 2011). We found that two different small GTPases, Rab11b and RhoA, are not involved in the mechanism that...
underlie GHSR basal inhibition of CaV, supporting the idea that an internalization process is unlikely to mediate this effect. If CaV channels are retained in intracellular compartments, an open question is what happens to them. There are several reports demonstrating that reduced CaV trafficking is followed by increased channel degradation through the proteasome (Waithe et al., 2011; Marangoudakis et al., 2012; Altier et al., 2011). Interestingly, it has been shown that CaVβ is necessary for the increase of NeDD4-1-mediated CaV channel degradation through the proteasome and lysosomes (Rougier et al., 2011). One key difference between the findings described by Rougier et al. and us is that, according to Rougier and colleagues, CaV3 channels are not affected by NeDD4-1 (officially known as NEDD4) – even in presence of CaVβ, indicating that distinct mechanisms are involved in both processes. More research is needed to conclude which molecular players execute the effect of GHSR basal activation on CaV trafficking.

We have shown previously that constitutively active GHSR reduces the surface density of CaV2 channels (Lopez Soto et al., 2015). Here, we extended our study to other CaV channel subtypes to show that this chronic basal inhibition by GHSR is common to all CaV subtypes, including neuronal CaV1 currents. CaV1 channels control CaV2 modulation of neurotransmission by coupling voltage changes to CaV2 influx at dendrites and soma of neurons (Dolmetsch et al., 2001; West et al., 2001). The best-studied effect of GPCR activity on CaV1 is the enhanced activity through acute activation of GPCRs (Olson et al., 2005). In our case, the number of CaV1 channels is chronically reduced – an effect that would compete with CaV2 release from internal compartments that has been described in response to GHSR activation in neurons (Cabral et al., 2012; Cowley et al., 2003; Andrews et al., 2009) – indicating that GHSR exert a fine control of CaV2 dynamics and, consequently, CaV2-dependent gene activation in neurons.

We also demonstrated that GHSR inhibits CaV3.2 currents. In neurons, CaV3 channels control the shape and frequency of action potentials (Perez-Reyes, 2003; Zhang et al., 2013), and changes in channel activity due to alternative splicing (Murbarian et al., 2004; Latour et al., 2004) or nonsense mutations (Powell et al., 2009) are responsible for pathophysiological states, such as epilepsy (Hamed, 2008). Yet, the mechanisms that control CaV3 trafficking and surface membrane stability are largely unknown (Zhang et al., 2013). What is known, however, is that hormonal changes during epilepsy can alter the surface expression of CaV3.1 (Qiu et al., 2006). Our current data suggest that neurons that express GHSR at high levels negatively modulate the participation of CaV3 in waveform and frequency of action potentials. A crucial finding is that the presence of CaVβ is mandatory for GHSR-mediated negative modulation of CaV3 currents. In this regard, the interaction between CaVβ and CaV3 is not clearly established. Some reports that support this interaction have shown that CaV3 currents are enhanced following coexpression of CaVβ (Dolphin et al., 1999; Dubel et al., 2004; Dolphin, 2003). By contrast other studies have failed to demonstrate a direct impact of CaVβ on CaV3 current levels (Leuranguer et al., 1998; Bae et al., 2010). Our data indicate for the first time that CaVβ is required for impairment of CaV3.2 trafficking mediated by constitutive active GPCR, thereby uncovering a new inhibitory function of CaVβ on CaV3 currents.

GHSRs are widely expressed in the brain (Zigman et al., 2006; Mani et al., 2014). On the basis of our current and previous data; we propose that this receptor controls CaV density in neurons. Moreover, we propose that impairment of CaV trafficking by GHSR can be overcome.
Materials and Methods

Cell culture and transient transfection
tsa201 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) with 10% fetal bovine serum (FBS; Internegocios) and subcultured at >10 passages to block the inhibitory effect of GHSR on CaV2.2 activity. CaV2.2W391A, CaV2.2, tsA201 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) with 10% fetal bovine serum (FBS; Internegocios) and subcultured at >10 passages to block the inhibitory effect of GHSR on CaV2.2 activity.

**A**  
CaV2.2W391A + CaVβδ1 + CaVβ2  
GHSR + GHSR  
+10 pA/pF  
5 ms  
-100  
10  
30  
CaV2.2  
GHSR  
+  
- 
-100  
10  5 ms  
-10  
10  
CaV2.2W391A + CaVβδ1 + CaVβ2  
GHSR + GHSR  
+10 pA/pF  
5 ms  
-100  
10  
30  
CaV2.2  
GHSR  
+  
-  
-100  
10  
5 ms  
-10  
10

**B**  
CaV2.2 + CaVβδ1 + CaVβ2TF8n  
GHSR + GHSR  
+10 pA/pF  
5 ms  
-100  
10  
20  
CaV2.2  
GHSR  
+  
-  
-100  
10  
5 ms  
-10  
10

Fig. 8. A W391A mutation of CaVβδ1 or truncation of CaVβ2 are not sufficient to block the inhibitory effect of GHSR on CaV2.2 activity. (A) Representative CaV current traces from tsA201 cells co-transfected with CaV2.2W391A, CaVβδ1, CaVβ2 and GHSR (+GHSR, n=7) or from controls transfected with empty plasmid (-GHSR, n=7), and average Ica for each condition (top). Representative CaV current traces from tsA201 cells co-transfected with CaV2.2W391A, CaVβδ1, CaVβ2 and GHSR (+GHSR, n=5) or from controls transfected with empty plasmid (-GHSR, n=5), and average Ica for each condition (bottom). (B) Representative CaV current traces from tsA201 cells co-transfected with CaV2.2, CaVβδ1, CaVβ2TF8n and GHSR (+GHSR, n=10) or from controls transfected with empty plasmid (-GHSR, n=14), and average Ica for each condition. Error bars represent means±s.e.m., individual points represent current registered. Student's t-test (top) and Mann-Whitney test (bottom).

Drugs

For patch-clamp and imaging experiments on tsA201 cells, the inverse GHSR agonist, [D-Arg1,D-Phε5,D-Trp7,9,Leu1]-substance P (SPA; Santa Cruz Biotechnology, Inc.) was used. For patch-clamp on mouse neuronal primary culture, CaV1 agonist Bay K 8644 (Sigma-Aldrich) was used.

Animals

Wild type and GHSR1a-deficient (GHSR-null) mice (3–5-month-old females) were bred at the IMBICE animal facility. Wild-type mice, on a pure C57BL/6 genetic background. GHSR-null mice, which fail to express the GHSR, were generated in D.L.’s lab. CaV3.2-GFP clone was a gift from Dr G. W. Zamponi (Department of Physiology and Pharmacology, University of Calgary, Canada). Auxiliary subunits CaVβ2TF8n and CaVβ3-eGFP were a gift from Dr M. V. Khvotchev (Department of Neurology, University of California, San Francisco, CA) and the pCDNA3-C3-toxin (C3-toxin) was provided by Dr. C. Davio (University of Buenos Aires, Argentina).

Mouse primary neuron culture

Neuron cultures were obtained from GHSR-null mice at embryonic days 16–18. The protocol used was similar to the same described by Raingo et al.
In brief, the necks of pregnant mice were dislocated and embryos quickly removed. The brain of the embryo was exposed, placed on its dorsal side and the the hypothalamus was removed with forceps. Brains were placed in sterile Hank’s solution and rinsed twice. Then, cells were dissociated at 37°C for 20 min with 0.25 mg/ml trypsin (Microvet). Enzyme digestion was stopped by addition of 300 μl FBS, and 0.28 mg/ml deoxyribonuclease I from bovine pancreas (Sigma-Aldrich) was added. Cells were mechanically dissociated using several glass pipettes with consecutive smaller-tip diameters. We plated about 50,000 cells on 12-mm diameter glass coverslips that had previously been treated with poly-L-lysine (Sigma-Aldrich) and were laid over 24-well plates. We incubated cells at 37°C in a 95% air and 5% CO2 atmosphere with DMEM (Microvet)/F12 (1:1), supplemented with B27 (1:50, Gibco), 10% FBS, 0.25% glucose, 2 mM glutamine (Gibco), 3.3 µg/ml insulin (Novo Nordisk Pharmaceutical Industries, Inc.), 40 µg/ml gentamicin sulfate (Richter) and a 1% vitamin solution (Microvet). At day 4 of culture, half of the medium was replaced by glutaMax (Thaler et al., 2004).

**Electrophysiology**

Ion channel currents were recorded by using an Axopatch 200 amplifier (Molecular Devices). Data were sampled at 20 kHz and filtered at 10 kHz (~3 dB) using PCLAMP8.2 software (Molecular Devices). Recording electrodes with resistances between 2 and 4 MΩ were used and filled with internal solution. Series resistances of <6 MΩ were admitted and compensated to 80% with a 10 μs lag time. Current leak was subtracted on-line using a P/4 protocol. All recordings were obtained at room temperature (23°C).

**Ca2+ currents of transiently transfected tsA201 cells**

Whole-cell patch-clamp recordings were performed on transfected (GFP-positive) tsA201 cells. Internal pipette solution contained (in mM): 134 CsCl, 10 EGTA, 1 EDTA, 10 HEPES pH 7.4 and 4 MgATP, with CsOH. External solution contained (in mM): 2 CaCl2, 1 MgCl2, 10 HEPES pH 7.4 and 140 choline chloride, with CsOH. Some experiments were made using BaCl2 (10 mM or 20 mM) instead of CaCl2 to amplify CaV current amplitude. Cells were held at −100 mV to remove closed-state inactivation (Thaler et al., 2004).

The test-pulse protocol consisted of voltage square pulses that were applied every 10 s; CaV1.2: −100 mV to +10 mV for 15 ms, CaV2.2: −100 mV to +10 mV 25 ms, CaV1.3: −100 mV to −10 mV for 15 ms, and CaV3.2: −100 mV to −20 mV for 200 ms.

**Ba2+ currents of primary neuronal cultures**

Mouse neurons that had been cultured for 7–15 days were patched in voltage-clamp whole-cell mode at a holding potential of ~80 mV applying squared test pulses to 0 mV for 20 ms every 10 s (Raingo et al., 2007). Internal pipette solution contained (in mM): 134 CsCl, 10 EGTA, 1 EDTA, 10 HEPES pH 7.2 and 4 MgATP, with CsOH. External solution contained (in mM): 2 NaCl, 4.7 KCl, 1.2 MgCl2, 2.5 CaCl2, 10 HEPES pH 7.4 and 10 glucose, with NaOH. After the whole cell configuration, CaV currents were recorded replacing the external solution by a high [Ba2+] solution containing (mM): 10 BaCl2, 110 choline chloride, 20 tetraethylammonium chloride, 1 MgCl2, 10 HEPES pH 7.4, 10 glucose and 0.001 tetrodotoxin (TTX; Sigma-Aldrich), with CsOH.

**Imaging**

In experiments presented in Figs 2–4, tsA201 cells had been co-transfected with CaV2.2-GFP or CaV3.2-GFP, with or without its auxiliary subunits, GHSR or pcDNA3.1 (+). At 48 or 24 h after transfection of CaV3.2-GFP or CaV2.2-GFP, respectively, culture medium was replaced by 1 ml of 1 µg/ml of membrane marker solution (CellMask orange plasma membrane stain; Molecular Probes) and cells were kept at 37°C for 1 min. After that, cells were rinsed three times with 1× phosphate-buffered saline (PBS). To finish, PBS was removed and a clean coverslip was placed over the cell layer.

Fluorescence photomicrographs were obtained using an optical fluorescence microscope (Eclipse 50i; Nikon), equipped with B2A and G2A filters and a camera (DS-Ri1; Nikon). Photomicrographs were analyzed with FIJI free software, using the CellMask red signal to mark out the plasma membrane and quantify green fluorescence intensity in both the internal area (excluding plasma membrane) and the total area of each cell as integrated density. The fluorescence intensity corresponding to the membrane (membrane fluorescence) was calculated as the difference between the fluorescence corresponding to the total (total fluorescence) and the internal area. Finally, the CaV2.2-GFP or CaV3.2-GFP membrane fluorescence (in percent) was calculated for each cell, by using: (membrane fluorescence/total fluorescence)×100.

In experiments presented in Figs 5–7 tsA201 cells had been plated on glass coverslips treated previously with poly-L-lysine and laid over 12-well plates. Cells were co-transfected 24 h later with CaV1.2-eGFP or CaV1.3-eGFP and with different plasmid combinations by using Lipofectamine. Further 24 h later, cells were rinsed with 1×PBS. After that, cells were fixed with 4% paraformaldehyde in PBS for 20 min, followed by a rinse with 1×PBS twice and were mounted on glass slides using VECTASHIELD® mounting medium.

Confocal images were collected using a Zeiss LSM 800 confocal microscope and ZEN software. Quantification of colocalization of CaV1.2-eGFP or CaV1.3-eGFP with the different intracellular compartment markers was performed using Just another Colocalization Plugin (JaCoP) from FIJI, to calculate Manders’ overlap coefficient for each marker (CaVβ-β/GFP/ marker overlap).

**Statistics**

Data were analyzed and visualized by using the OriginPro 8 (Origin-Lab) and GraphPad Prism 5 (GraphPad Software, Inc.) software. We used the Kolmogorov–Smirnov to test for conformity to a normal distribution; variance homogeneity was examined by using Bartlett’s (normal distributed data) and Brown-Forsythe’s (no normal distributed data) test. P values were calculated from one- or two-sample t-tests (normally distributed data) or Mann–Whitney test (no normally distributed data), and multiple comparison one-way-ANOVA with Tukey’s post hoc test (normal distributed data) or non-parametric Kruskal–Wallis test with Dunn’s post-test (no normal distributed data). P values were evaluated and included in the figures. Specific statistical test used is indicated for each data set. Data were expressed as mean±s.e.m.

**Acknowledgements**

We thank Dr Jeffrey Zigman (The University of Texas Southwestern Medical Center, Dallas, TX) for providing GHSR-null mice and Dr Mario Perello and Guadalupe Garcia Romero (both Multidisciplinary Institute of Cellular Biology (IMBICE), Universidad Nacional de La Plata, Buenos Aires, Argentina) for help and advice in housing and caring for the mouse colony. We thank Sylvia Denome for her excellent technical assistance and Daniel Dubreuil (both Department of Neuroscience, Brown University, Providence, RI) for kindly helping us with confocal microscopy.

**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**


**Funding**

This work was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) (PICT2013-1145 and PICT2015-3330 to J.R.). E.R.M., V.M.D., S.S.R. and J.R. were supported by Consejo Nacional de Investigaciones Científicas y Comisiones de Investigaciones de la Provincia de Buenos Aires (CIC).

**References**


