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Growth hormone secretagogue receptor constitutive activity impairs voltage-gated calcium channel-dependent inhibitory neurotransmission in hippocampal neurons

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Key points

- Presynaptic Ca_V2 voltage-gated calcium channels link action potentials arriving at the presynaptic terminal to neurotransmitter release. Hence, their regulation is essential to fine tune brain circuitry.
- Ca_V2 channels are highly sensitive to G protein-coupled receptor (GPCR) modulation. Our previous data indicated that growth hormone secretagogue receptor (GHSR) constitutive activity impairs Ca_V2 channels by decreasing their surface density.
- We present compelling support for the impact of $Ca_V2.2$ channel inhibition by agonist-independent GHSR activity exclusively on GABA release in hippocampal cultures. We found that this selectivity arises from a high reliance of GABA release on $Ca_V2.2$ rather than on $Ca_V2.1$ channels.
- Our data provide new information on the effects of the ghrelin–GHSR system on synaptic transmission, suggesting a putative physiological role of the constitutive signalling of a GPCR that is expressed at high levels in brain areas with restricted access to its natural agonist.

Abstract Growth hormone secretagogue receptor (GHSR) displays high constitutive activity, independent of its endogenous ligand, ghrelin. Unlike ghrelin-induced GHSR activity, the physiological role of GHSR constitutive activity and the mechanisms that underlie GHSR neuronal modulation remain elusive. We previously demonstrated that GHSR constitutive activity modulates presynaptic Ca_V2 voltage-gated calcium channels. Here we postulate that GHSR constitutive activity-mediated modulation of Ca_V2 channels could be relevant in the hippocampus since this brain area has high GHSR expression but restricted access to ghrelin. We performed whole-cell patch-clamp in hippocampal primary cultures from E16- to E18-day-old C57BL6 wild-type and GHSR-deficient mice after manipulating GHSR expression with lentiviral transduction. We found that GHSR constitutive activity impairs $Ca_V2.1$ and $Ca_V2.2$ native calcium currents and that $Ca_V2.2$ basal impairment leads to a decrease in GABA but not glutamate release.

Valentina Martínez Damonte is a PhD student in the Electrophysiology Laboratory of the Multidisciplinary Institute of Cellular Biology (IMBICE) in La Plata, Buenos Aires. Her current work is focused on voltage-gated calcium channel modulation and her primary interest is to continue studying the mechanisms that underlie synaptic transmission.

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We postulated that this selective effect is related to a higher $Ca_V2.2$ over $Ca_V2.1$ contribution to GABA release (∼40% for $Ca_V2.2$ in wild-type *vs.* ∼20% in wild-type GHSR-overexpressing cultures). This effect of GHSR constitutive activity is conserved in hippocampal brain slices, where GHSR constitutive activity reduces local GABAergic transmission of the granule cell layer (intra-granule cell inhibitory postsynaptic current (IPSC) size ∼−67 pA in wild-type *vs.* ∼−100 pA in GHSR-deficient mice), whereas the glutamatergic output from the dentate gyrus to CA3 remains unchanged. In summary, we found that GHSR constitutive activity impairs IPSCs both in hippocampal primary cultures and in brain slices through a Ca_V2-dependent mechanism without affecting glutamatergic transmission.

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Introduction

Growth hormone secretagogue receptor (GHSR; or growth hormone secretagogue type 1a receptor) is a G protein-coupled receptor (GPCR) with two different activation modes: one constitutive and the other agonist-induced (Holst *et al.* 2003). The relevance of its ghrelin-induced active mode in energy balance and in hedonic and addictive aspects of eating has been well studied (Perello *et al.* 2010; Abizaid, 2011; Henderson *et al.* 2013; Hsu, 2015; Perello & Dickson, 2015). On the other hand, the function of GHSR's constitutively active mode is less understood due to its chronic nature and the scarcity of specific inverse agonists (Holst *et al.* 2007). Few studies have unmasked a clear physiological role of GHSR constitutive activity. In this regard, the influence of this chronic active receptor on overeating behaviour after fasting in mice has recently been shown (Fernandez *et al.* 2018). At the cellular level we have demonstrated that GHSR constitutive activity severely reduces presynaptic voltage-gated calcium (Ca_V) channel trafficking to the plasma membrane (Lopez Soto, 2015; Mustafá *et al.* 2017) by promoting the retention of the Ca_V complex in the endoplasmic reticulum. Ca_V channels generate the calcium influx that is triggered by depolarization in excitable cells. In particular $Ca_V2.1$ and $Ca_V2.2$ channels are responsible for calcium-induced neurotransmitter release at presynaptic terminals (Catterall & Few, 2008). Because of their critical role in neurotransmission, there are many physiological mechanisms aimed at controlling Ca_V2 activity, GPCR activation being one of the most effective. GPCR-mediated modulation of Ca_V channels can lead to dramatic changes in channel kinetics and in membrane channel density (Zhang *et al.* 2008; Zamponi & Currie, 2013). Thus, we reason that inhibition of Ca_V channels by GHSR constitutive activity could be relevant in brain areas with high GHSR expression level but restricted access to ghrelin (Cabral *et al.* 2014, 2015).

The hippocampus is a highly plastic glutamatergic neuronal circuit influenced by many diverse GABAergic inhibitory interneurons (Maccaferri & Lacaille, 2003; Houser, 2007; Mann & Paulsen, 2007). This area is far from the sites that ghrelin utilizes to access the brain, such as the median eminence and the circumventricular organs (Cabral *et al.* 2015, 2017; Abizaid & Edwards, 2017). Despite this, GHSR is widely expressed at relatively high levels in the hippocampus, particularly in the dentate gyrus (Zigman *et al.* 2006; Cabral *et al.* 2013; Mani *et al.* 2014, 2017), where exogenous ghrelin application modulates behavioural responses such as the reward value of food, response to stress, memory retention, spatial memory and learning behaviour (Diano *et al.* 2006; Atcha *et al.* 2009; Carlini *et al.* 2010; Chen *et al.* 2011; Davis *et al.* 2011; Wang, 2013; Cahill *et al.* 2014; Zhao *et al.* 2014; Kanoski & Grill, 2015; Kent *et al.* 2015; Hsu *et al.* 2016). The molecular mechanisms implicated in these effects include dendritic spine formation and long-term potentiation development (Diano *et al.* 2006), increased AMPA receptor trafficking (Ribeiro *et al.* 2014) and enhanced activity of NMDA receptors (Cuellar & Isokawa, 2011; Ghersi *et al.* 2015).

Despite that ghrelin-mediated effects at the hippocampus mainly target glutamatergic neurons, GHSR is expressed in glutamatergic but also in GABAergic neurons in several brain regions. The areas where GABA neurons are influenced by ghrelin action include the hypothalamus (Wang, 2013; Lopez Soto, 2015), the area postrema (Cabral *et al.* 2017) and the central nucleus of the amygdala (Cruz *et al.* 2012).

In contrast, the effect of GHSR constitutive activity on the brain remains unclear. Here we aimed to study the effect of GHSR constitutive activity on presynaptic Ca_V currents and synaptic transmission in neurons from the hippocampus, a brain area with restricted ghrelin access.

Methods

Ethical approval

All experimentation in this study received approval from the ethical committee of IMBICE in strict accordance with the recommendations of the *Guide for the Care and Use of Laboratory Animals* of the National Research Council, USA

and all efforts were made to minimize suffering. (Reference number from Ethical Committee of IMBICE: 03-09-16 – 'Caracterización de la modulación de los canales de calcio por receptores acoplados a proteína G'.) We understand the ethical principles under which *The Journal of Physiology* operates and ensured that our work complied with the policies and regulations regarding animal ethics.

Origin and source of the animals

This study was performed using wild-type (WT) mice on a pure C57BL/6 background and GHSR-deficient mice, which fail to express GHSR (Zigman *et al.* 2005) and were derived from crosses between heterozygous animals back-crossed >10 generations onto a C57BL/6 genetic background. Mice of both sexes were bred at the animal facility of the IMBICE. Mice were housed in a 12 h light/dark cycle in a climate-controlled room (22°C) with *ad libitum* access to water and food.

Mouse neuronal primary culture

Hippocampal neuronal cultures were obtained from WT and GHSR-deficient mice at embryonic days 16–18. A total of 17 pregnant females were subjected to cervical dislocation between days 16 and 18 of pregnancy in order to quickly remove embryos. Embryos were placed immediately in cold Hanks' solution. Embryos' brains were exposed and the hippocampi removed, placed in sterile Hanks' solution and rinsed twice. Then, cells were dissociated at 37°C for 20 min with 0.25 mg ml^{-1} trypsin (Microvet, Buenos Aires, Argentina). Next, 300 μl of fetal bovine serum (FBS, Internegocios, Mercedes, Buenos Aires, Argentina) to stop the enzyme digestion and 0.28 mg ml^{-1} deoxyribonuclease I from bovine pancreas (Sigma-Aldrich, Buenos Aires, Argentina) were added. Cells were mechanically dissociated using several glass pipettes with consecutive smaller-tip diameters. About 60,000 cells were plated on 12 mm-diameter glass coverslips treated previously with poly-L-lysine (Sigma-Aldrich) and laid over 24-well plates. Cells were incubated at 37°C in a 95% air −5% CO_2 atmosphere with Dulbecco's modified Eagle's medium/F12 (DMEM/F12) (Microvet) 1:1 medium supplemented with B27 supplement (1:50, Gibco, Thermo Fisher Scientific, Buenos Aires, Argentina), 10% FBS, 0.25% glucose, 2 mM glutamine (Gibco), 3.3 μg ml^{-1} insulin (Novo Nordisk Pharmaceutical Industries, Inc., Buenos Aires, Argentina), 40 μg ml^{-1} gentamicin sulfate salt (Richet, Buenos Aires, Argentina), and 1% vitamin solution (Microvet). On the fourth day in culture, half of the incubating medium was replaced with fresh medium containing cytosine β-D-arabinofuranoside (Sigma-Aldrich) to reach a final concentration of 5 μM.

Lentiviral trasduction

tsA201 cells were grown in DMEM (Gibco) with 10% FBS and subcultured when 80% confluent. tsA201 cells were plated in a 100 mm-diameter dish; 24 h later they were co-transfected with plasmids coding for a third generation lentiviral system using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Buenos Aires, Argentina) reagent. The culture medium was changed 1 h prior to transfection. A total of 20 mg of plasmids DNA was used for the transfection of one dish: 3.5 mg of the envelope plasmid pCMV-VSV-G, 6.5 mg of packaging plasmids pMDLg/pRRE and pRSV-Rev, and 10 mg of transfer vector plasmid containing GHSR or a natural mutant lacking constitutive activity, GHSRA204E. Conditioned medium was collected after 24 h, cleared by low-speed centrifugation, filtered through 0.22 μm pore size filters and used to replace growth medium. After transfection, cells were kept in culture to allow lentiviral production for 48 h. Then, the medium was collected and centrifuged at room temperature at 1000 rpm. The supernatant was filtered through 0.45 μm pore size filters and 500 μl was used to replace the growth medium of each well containing the primary neuron culture for 4 h.

Hippocampal slice preparation

Adult WT and GHSR-deficient mice were anaesthetized with isofluorane (2%) and immediately decapitated. Brains were quickly removed and hippocampal horizontal brain slices (300 μm) containing the hippocampus (Bischofberger *et al.* 2006; Xiong *et al.* 2017) were prepared in bubble ice-cold 95% O_2–5% CO_2-equilibrated solution containing (in mM): 110 choline chloride, 25 glucose, 25 $NaHCO_3$, 7 $MgCl_2$, 11.6 ascorbic acid, 3.1 sodium pyruvate, 2.5 KCl, 1.25 NaH_2PO_4 and 0.5 $CaCl_2$. Slices were then stored at room temperature in 95% O_2–5% CO_2-equilibrated artificial cerebrospinal fluid (aCSF) containing (in mM): 124 NaCl, 26.2 $NaHCO_3$, 11 glucose, 2.5 KCl, 2.5 $CaCl_2$, 1.3 $MgCl_2$ and 1 NaH_2PO_4.

Drugs

In some patch-clamp recordings on mouse neuronal primary culture the $Ca_V2.1$ blocker ω-agatoxin-IVA (0.2 μM, Peptides International, Louisville, KY, USA) and the $Ca_V2.2$ blocker ω-conotoxin-GVIA (1 μM, Alomone Labs, Jerusalem, Israel) were used.

Electrophysiology

Ion channel currents were recorded with an Axopatch 200 (Molecular Devices, San Jose, CA, USA) or an EPC7 (HEKA Electonik, Lambrecht/Pfalz, Germany) amplifier. Data were sampled at 20 kHz and filtered at 10 kHz (−3 dB) using pCLAMP8.2 (Molecular Devices) or

PatchMaster (HEKA) software. Recording electrodes with resistances between 2 and 5 MΩ were used and filled with internal solution. Series resistances of less than 6 MΩ were admitted and compensated 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 (∼24°C).

Barium currents of primary neuronal cultures. Mouse-cultured neurons of 5–21 days *in vitro* (DIV) were patched-clamped 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 and 4 MgATP, pH 7.2 with CsOH. Neurons were bathed with high sodium external solution containing (in mM): 135 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 10 Hepes and 10 glucose, pH 7.4 with NaOH. After attaining the whole-cell configuration, Ca_V currents were recorded replacing the external solution by a high barium solution containing (in mM): 10 $BaCl_2$, 110 choline chloride, 20 tetraethylammonium chloride, 1 $MgCl_2$, 10 Hepes, 10 glucose and 0.001 tetrodotoxin (TTX; Sigma-Aldrich), pH 7.4 with CsOH.

Postsynaptic currents of primary neuronal cultures. Mouse neurons of 12–21 DIV were patch-clamped in voltage-clamp whole-cell mode at a holding potential of −80 mV. Internal pipette solution contained (in mM): 115 caesium methanesulfonate, 10 CsCl, 5 NaCl, 10 Hepes, 20 tetraethylammonium chloride, 4 Mg-ATP, 0.3 NaGTP, 0.6 EGTA and 10 lidocaine *N*-ethyl bromide (pH 7.2 with CsOH). The external solution used was the high sodium solution described above, containing 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM Alomone Labs) and (2*R*)-amino-5-phosphonopentanoate (APV, 100 μM Alomone Labs) or picrotoxin (50 μM, Sigma-Aldrich) in order to isolate inhibitory or excitatory postsynaptic currents, respectively (IPSCs and EPSCs). Electrical stimulation through parallel platinum electrodes (duration, 1 ms; amplitude, 20 mA) was delivered while neurons were held at −80 mV to elicit evoked responses. TTX (1 μM) was added in order to record miniature IPSCs and EPSCs (mIPSCs and mEPSCs). For Fig. 3, paired-pulse responses were recorded delivering paired electrical stimulation and varying the inter-stimulus space between 20 and 200 ms. For Fig. 4, the amount of $CaCl_2$ in the external solution was modified from 0.5 to 10 mM.

Hyperosmotic shock. Charge movement was recorded as a response to hyperosmotic shock, applied as a 0.5 M sucrose solution pulse while neurons were held at −80 mV. Average charge was measured as integrated area 3 s before and 3 s after peak response.

Postsynaptic currents from hippocampal slices. Recordings were made under a Zeiss Examiner.A1 microscope at 25°C in aCSF under a flow rate of 2.5 ml min^{-1}. Access resistance and input resistance were monitored by a step of −10 mV. Experiments were discarded if the access resistance increased by >20%. The internal solution used to examine neuronal excitability contained (in mM): 140 potassium gluconate, 5 NaCl, 5 KCl, 4 $MgCl_2$, 0.6 EGTA, 10 Hepes, 3 Na_2ATP and 0.3 Na_2GTP (pH 7.3 with KOH). Before recording postsynaptic currents, intrinsic properties of neurons were monitored by current-clamp. Both IPSCs from granule cells and EPSCs from CA3 pyramidal cells were evoked by a concentric bipolar electrode placed in the granule cell layer of the hippocampus and recorded in whole-cell voltage-clamp configuration. Cells were maintained at −80 mV throughout the voltage clamp recording.

Statistics

Data were analysed using the OriginPro 8 (Origin-Lab Corp., Northampton, MA, USA) and Prism 6 (GraphPad Software, Inc., San Diego, CA, USA) software. We used the Kolmogorov–Smirnov test to test for conformity to a normal distribution, and variance homogeneity was examined using Bartlett's (normally distributed data) and Brown–Forsythe's (non-normally distributed data) test. *P* values were calculated from Student's *t* test or multiple comparison one-way ANOVA with Tukey's *post hoc* test (normally distributed data), or from the Mann–Whitney test or the Kruskal–Wallis test with Dunn's *post hoc* test (non-normally distributed data), after trying data transformation. The specific statistical test used and sample size are indicated for each data set in the figure legends. Data were expressed as mean ± SEM, and individual data points are represented as black dots.

Results

To assess whether GHSR constitutive activity could impact Ca_V currents in hippocampal neurons we performed voltage-clamp whole-cell recordings in primary cultured neurons from WT and GHSR-deficient mice. We manipulated GHSR levels by infecting the cultures with lentiviruses expressing GHSR tagged with green fluorescent protein (GFP). We found that the total Ca_V current in hippocampal neurons significantly increased after 7 DIV (Fig. 1*A*) and that GHSR overexpression occluded the Ca_V current increase in both genotypes (between 5 and 14 DIV). Notably, the increase in current amplitude was higher in neurons from the GHSR-deficient cultures than from the WT cultures (>14 DIV, Fig. 1*B*) and GHSR overexpression decreased Ca_V current levels to the same extent in both conditions. To confirm that the reduction of Ca_V current was due to GHSR

ligand-independent activity, we overexpressed a mutant version of GHSR, GHSRA204E, which lacks constitutive activity but signals upon ghrelin binding (Pantel *et al.* 2006; Inoue *et al.* 2011). We found that Ca_V current levels in neurons overexpressing GHSRA204E are not different from the WT condition (Fig. 1*B*). Hence, our approach dismisses a possible effect of a ligand mediating this Ca_V current impairment. Altogether our results indicate that GHSR expression reduces Ca_V current levels in hippocampal neurons and that this effect is related to its constitutive activity.

Since Ca_V channels couple depolarization to neurotransmitter release, we explored the effect of GHSR expression on Ca_V-dependent synaptic activity. We measured the amplitude of EPSCs and IPSCs evoked by field stimulation in mature hippocampal cultures from WT and GHSR-deficient mice. We found that EPSC amplitude was unaffected by GHSR or GHSRA204E overexpression in WT and GHSR-deficient cultures (Fig. 2*A*). In contrast, IPSC amplitude was significantly reduced by GHSR expression in WT and GHSR-deficient cultures (Fig. 2*B*). Additionally, in WT cultures, IPSC amplitude was unaffected by overexpression of GHSRA204E. These results indicate that GHSR expression exerts selective inhibitory effect on GABAergic neurotransmission. Next, we reasoned that Ca_V channels were likely involved in the GHSR-dependent GABAergic neurotransmission impairment since GHSR constitutive activity reduces Ca_V currents (Figs. 1 and 2; Lopez Soto, 2015). We used different approaches to identify the specific targets of GHSR on inhibitory neurotransmission in hippocampal cultures. We estimated release probability, a presynaptic activity feature, in GHSR-deficient and WT hippocampal cultures overexpressing or not overexpressing GHSR by measuring the ratio of two postsynaptic currents elicited by two consecutive pulses with different time intervals. We found that excitatory paired pulse ratios (PPR) were similar among GHSR-deficient, WT and WT overexpressing GHSR cultures at any inter-pulse time assayed (between 20 and 200 ms; Fig. 3*A*). In contrast, inhibitory PPR was decreased in GHSR-deficient cultures in comparison with cultures overexpressing GHSR at inter-pulse intervals shorter than 50 ms (Fig. 3*B*). This change could be attributed to the decrease in

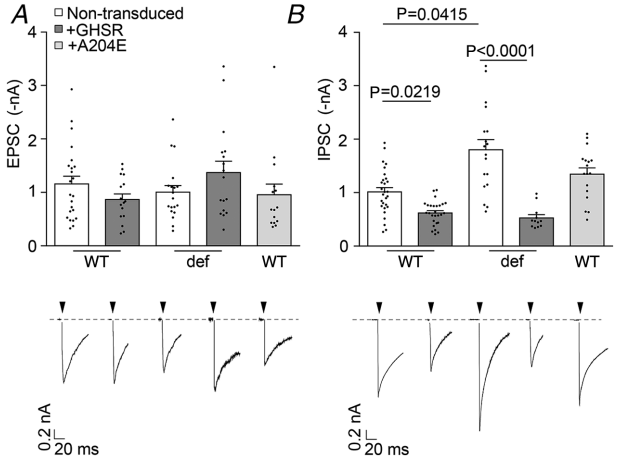

Figure 2. GHSR constitutive activity impairs Ca_V-dependent GABAergic transmission while fails to modify glutamatergic transmission
EPSC (*A*) and IPSC (*B*) evoked by electrical stimuli (indicated by arrowheads). Representative traces and average values from mature (>14 DIV) wild-type (WT) and GHSR-deficient (def) hippocampal neurons transduced with lentiviral plasmids encoding GHSR (+GHSR) or GHSRA204E (+A204E) or not transduced. Statistical significance evaluated by Kruskal–Wallis ANOVA and Dunn's *post hoc* test.

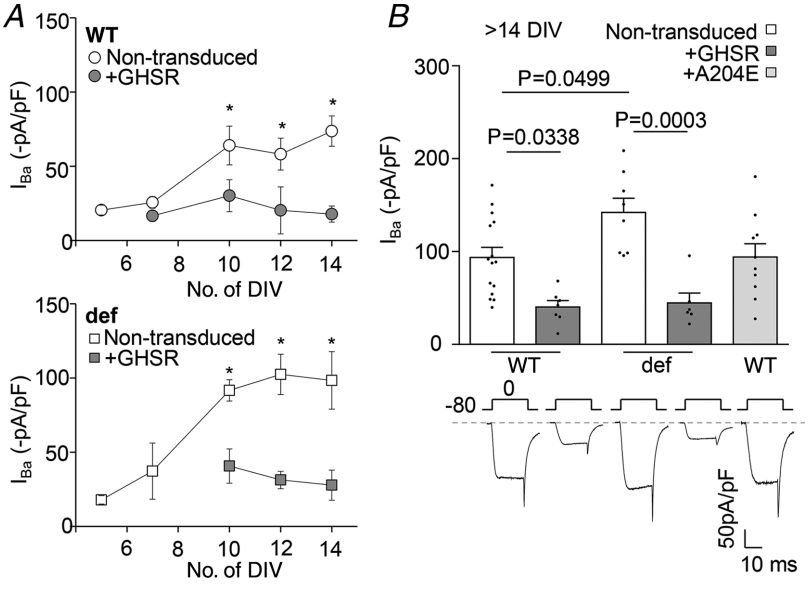

Figure 1. GHSR constitutive activity affects native I_{CaV} levels in hippocampal cultured neurons
A, average I_{Ba} levels from 5–14 DIV wild-type (WT) and GHSR-deficient (def) hippocampal neurons transduced with lentiviral plasmids encoding GHSR (+GHSR) or GHSRA204E (+A204E) or not transduced. Statistical significance evaluated by Mann–Whitney test (n = 122, 3–12 per mean data point). *B*, representative traces and average I_{Ba} levels from mature (>14 DIV) hippocampal neurons in the same conditions as in *A*. Statistical significance evaluated by ANOVA and Tukey's *post hoc* test.

presynaptic Ca_V2 channel density and, in consequence, to more synaptic vesicles remaining available for release by the second pulse when GHSR is expressed. Notably, this PPR difference between GHSR-deficient and GHSR-expressing cultures disappeared at longer inter-pulse intervals (Fig. 3*B*), as expected since a longer time between pulses would allow synaptic vesicle pool replenishment in the GHSR-deficient condition. We further characterized the calcium sensitivity of neurotransmitter release in hippocampal GHSR-expressing or GHSR-deficient cultures. We recorded EPSC and IPSC at five different external calcium concentrations ranging from 0.5 to 10 mM in WT and GHSR-deficient cultures. While we found no differences in EPSC curves (Fig. 4*A*), we observed that the *y*-intercept of the IPSC size *versus* external calcium concentration curve in GHSR-deficient neurons was significantly shifted from about −0.5 to −0.8 nA (Fig. 4*B*). This suggests that the number of presynaptic Ca_V available for coupling the depolarization to synaptic vesicle release is smaller when GHSR is expressed in GABAergic neurons. Moreover, the IPSC *versus* calcium concentration curve slopes in WT and GHSR-deficient cultures were the same (pooled slope = 0.501558, Fig. 4*B*) indicating that the intrinsic ability of presynaptic Ca_V channels to trigger neurotransmission is unchanged by GHSR expression. Additionally, we assayed the effect of GHSR expression on Ca_V-independent neurotransmitter release by two experimental paradigms. First we tested the frequency of excitatory and inhibitory miniature spontaneous events (mEPSC and mIPSC) in the presence of 100 μM of cadmium chloride, a specific Ca_V blocker, and found no differences among conditions (Fig. 5). We also found no differences in the current mobilized by neurotransmitter release (GABA or glutamate) evoked by a hyperosmotic solution (500 mM sucrose, Fig. 6). Taken together our results show that GHSR constitutive activity reduces presynaptic Ca_V density, decreasing probability of GABA release, while glutamate release is not affected, indicating a presynaptic mechanism exclusive for inhibitory neurotransmission impairment.

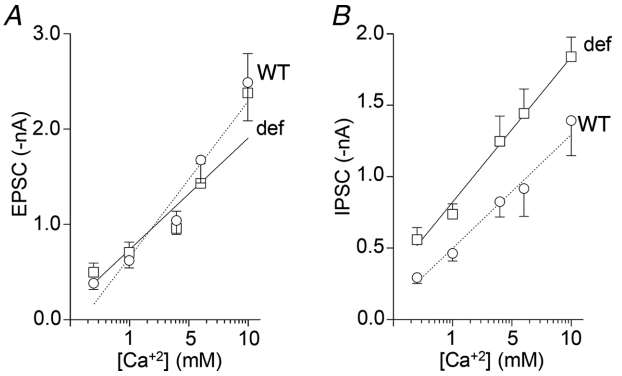

Figure 4. Increased IPSC size due to the absence of GHSR is conserved at different Ca^{2+} concentrations
EPSC (*A*) and IPSC (*B*) average values at different Ca^{2+} concentrations (logarithmic scale) and linear regression plot from >14 DIV wild-type (WT, open circles) and GHSR deficient (def, open squares) hippocampal neurons. Linear regression fitting parameters: EPSC slope test: $F = 1.22978$, degree of freedom for numerator (DFn) = 1, degree of freedom for denominator (DFd) = 75, $P = 0.271$, pooled slope = 0.576134 r^2 (WT) = 0.7167, r^2 (def) = 0.5260; intercepts test: $F = 0.0203306$, DFn = 1, DFd = 76, $P = 0.887$, pooled intercept = 2.77994. ($n = 79$, ranging between 3–12 per mean data point.) IPSC slope test: $F = 0.699603$, DFn = 1, DFd = 81, $P = 0.4054$, pooled slope = 0.501558, r^2 (WT) = 0.5170, r^2 (def) = 0.5888; intercepts test $F = 29.3249$, DFn = 1, DFd = 82, $P < 0.0001$, intercept (WT) = −0.50 ± 0.05 nA, intercept (def) = −0.82 ± 0.06 nA. ($n = 85$, ranging between 3–13 per mean data point.)

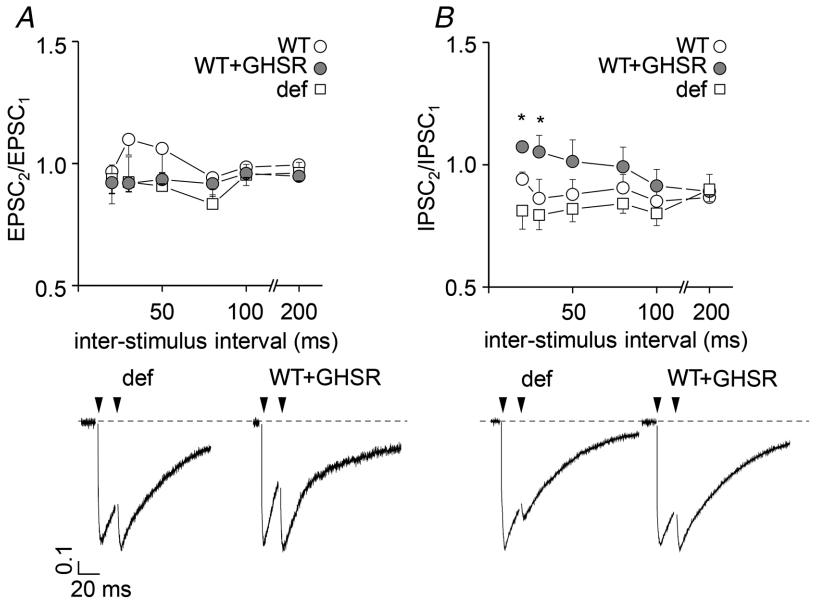

Figure 3. Paired pulse ratio (PPR) is modified by GHSR expression levels only for GABAergic transmission
Average EPSC (*A*) and IPSC (*B*) PPR at different inter-stimulus intervals (20–200 ms) from >14 DIV GHSR-deficient (def) and wild-type (WT) hippocampal neurons transduced with lentiviral plasmids encoding GHSR (WT+GHSR) or not (WT) and examples of paired pulses for WT overexpressing GHSR (WT+GHSR) and GHSR deficient (def) conditions evoked by 1 ms depolarizing stimuli at a 20 ms inter-stimulus interval. Electrical stimuli are indicated by arrowheads. Statistical significance evaluated by Kruskal–Wallis ANOVA and Dunn's *post hoc* test ($n = 283$, 4–11 per mean data point).

To test if there is a Ca_V subtype coupling preference to GABA or glutamate release underlying the specific GHSR effect on inhibitory neurotransmission, we characterized the $Ca_V2.1$ and $Ca_V2.2$ dependency of neurotransmitter release in hippocampal cultures expressing or not expressing GHSR. First, we assayed the effect of ω-conotoxin-GVIA, a specific inhibitor of $Ca_V2.2$, and ω-agatoxin-IVA, a specific inhibitor of $Ca_V2.1$, on Ca_V currents from neurons from mature GHSR-deficient, WT and WT overexpressing GHSR mature cultures (Fig. 7). We found that the amount of Ca_V current inhibited by ω-conotoxin-GVIA was smaller in WT (~30%) and GHSR-overexpressing neurons (~15%), compared with GHSR-deficient neurons (~45%), while the percentages of current inhibited by ω-agatoxin-IVA were the same in these conditions in comparison with GHSR-deficient neurons (~20%). These data suggest that GHSR constitutive activity impairs mainly $Ca_V2.2$ current in hippocampal neurons. Of note, consistent with our results (Fig. 1), we found a smaller total Ca_V current from WT neurons and GHSR-overexpressing neurons in comparison with GHSR-deficient neurons. Based on this result, we hypothesized that there is a $Ca_V2.2$ coupling preference to GABA rather than to glutamate release which in turn mediates the GHSR effect on inhibitory neurotransmission. Thus, we analysed the effect of ω-conotoxin-GVIA on IPSCs and EPSCs. We found a larger reliance of IPSC ($37.7 \pm 4.1\%$, $n = 9$) rather than of EPSC ($21.23 \pm 3.3\%$, $n = 7$) on $Ca_V2.2$ ($P = 0.0097$, Student's t test) in WT cultures. Additionally, as we previously showed, GHSR overexpression reduced IPSC amplitudes ~50% (Figs. 2 and 8*A* and *B*). IPSCs in WT cultures overexpressing GHSR were only ~20% ω-conotoxin-GVIA sensitive, while in WT cultures the ω-conotoxin-GVIA sensitive IPSCs were ~40% (Fig. 8*C*). Consistent with our hypothesis, IPSC reliance on $Ca_V2.1$ of both WT and WT overexpressing GHSR cultures was similar (~15%, Fig. 8*D*). Thus, $Ca_V2.1$ dependency of IPSC was significantly smaller than the $Ca_V2.2$ dependency in GHSR WT cultures and

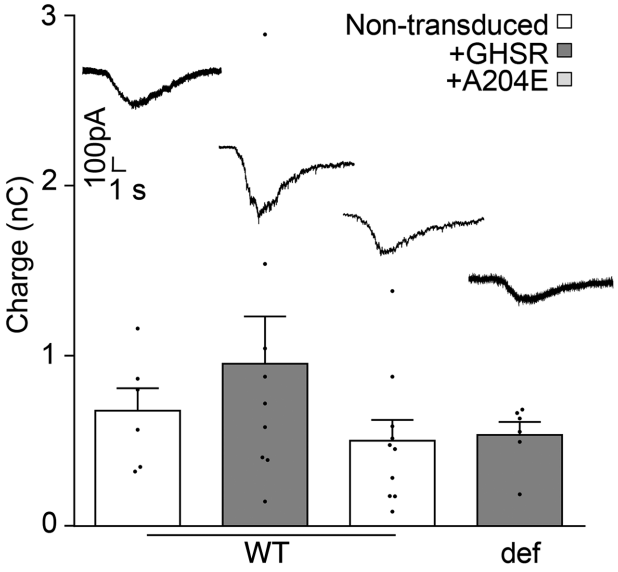

Figure 6. Hyperosmotic sucrose response is unaffected by GHSR expression
Representative traces and average mobilized charge values from mature (>14 DIV) wild-type (WT) and GHSR deficient (def) hippocampal neurons transduced with lentiviral plasmids encoding GHSR (+GHSR) and GHSRA204E (+A204E) or not transduced (non-transduced) in response to 0.5 M sucrose stimulation in the presence of 1 μM TTX and 100 μM Cd^{2+}. Statistical significance evaluated by Kruskal–Wallis ANOVA and Dunn's *post hoc* test.

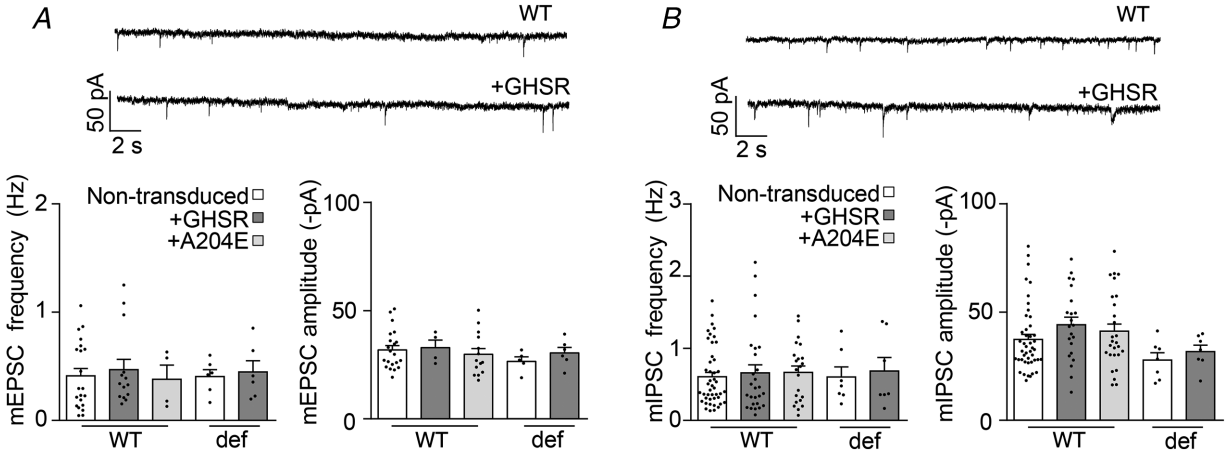

Figure 5. Ca_V-independent mEPSCs and mIPSC are unaffected by GHSR constitutive activity
mEPSC (*A*) and mIPSC (*B*) average amplitude and frequency values and representative traces from mature (>14 DIV) wild-type (WT) and GHSR-deficient (def) hippocampal neurons transduced with lentiviral plasmids encoding GHSR (+GHSR) and GHSRA204E (+A204E) or not transduced. Statistical significance evaluated by Kruskal–Wallis ANOVA and Dunn's *post hoc* test. Both mEPSC and mIPSC were recorded in the presence of 1 μM TTX and the Ca_V blocker 100 μM Cd^{2+}.

it remained unchanged when GHSR was overexpressed. Taken together our results suggest that GHSR constitutive activity specifically decreases inhibitory neurotransmission by impairing $Ca_V2.2$ currents that support GABA release in hippocampal cultures.

Finally, to confirm that our results obtained in primary cultures are conserved in intact adult hippocampal tissue, we performed electrophysiological recordings in acute horizontal brain slices from dentate gyrus granule cells and CA3 pyramidal neurons from GHSR-deficient and WT mice. In each experiment we monitored the intrinsic properties of both neuron types and we found no differences either in the resting membrane potential or in the firing voltage threshold between GHSR-deficient and WT slices (in mV: $V_{resting}$ WT: -68.50 ± 2.90, GHSR-deficient: -69.08 ± 4.53 from granule cells; WT: -57.27 ± 4.18, GHSR-deficient: -57.50 ± 2.50 from CA3 pyramidal cells; $V_{threshold}$ WT: -45.33 ± 2.04, GHSR-deficient: $-41.54 \pm .91$ from granule cells; WT: -48.75 ± 5.32, GHSR-deficient: -43.33 ± 1.67 from CA3 pyramidal cells. These values are in accordance with previous data showing that granule cells from the dentate gyrus display a characteristic low input resistance and a hyperpolarized resting membrane potential (Staley *et al.* 1992; Mongiat *et al.* 2009). As these cells are also subjected to strong inhibition by local interneurons (Dieni *et al.* 2013; Temprana *et al.* 2015), they display low firing frequencies at resting membrane potential (Krueppel *et al.* 2011).

In this experimental setting, we then recorded the magnitude of intra-dentate gyrus IPSCs (GC-IPSCs), using a stimulating electrode placed within the granule cell layer (Fig. 9*A*). We found that GC-IPSCs recorded from WT mice were significantly smaller than those from GHSR-deficient mice (Fig. 9*B*). In the same preparation, we explored if GHSR constitutive activity affects the excitatory output from the dentate gyrus to CA3, by measuring the amplitude of EPSCs in CA3 pyramidal neurons stimulating the granule cell layer (Fig. 9*C*). In line with our previous results, we found that GHSR constitutive activity fails to modify excitatory transmission.

Altogether, these last experiments indicate that the presence of GHSR in the dentate gyrus is capable of modulating the inhibitory transmission in intact hippocampal circuitry.

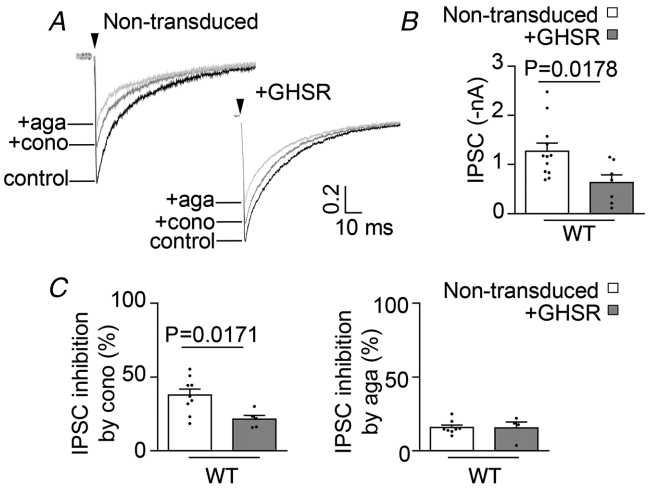

Figure 8. GHSR expression inhibition of GABAergic transmission is related to a higher reliance on $Ca_V2.2$
A, IPSC representative traces before (control) and after successive application of 1 μM ω-conotoxin-GVIA (+cono) and 0.2 μM ω-agatoxin-IVA, *B* and *C*, average basal levels (*B*) and percentage of inhibition (*C*) from >12 DIV wild-type (WT) hippocampal neurons and wild-type hippocampal neurons transduced with lentiviral plasmids encoding GHSR (+GHSR). Statistical significance evaluated by Student's *t* test.

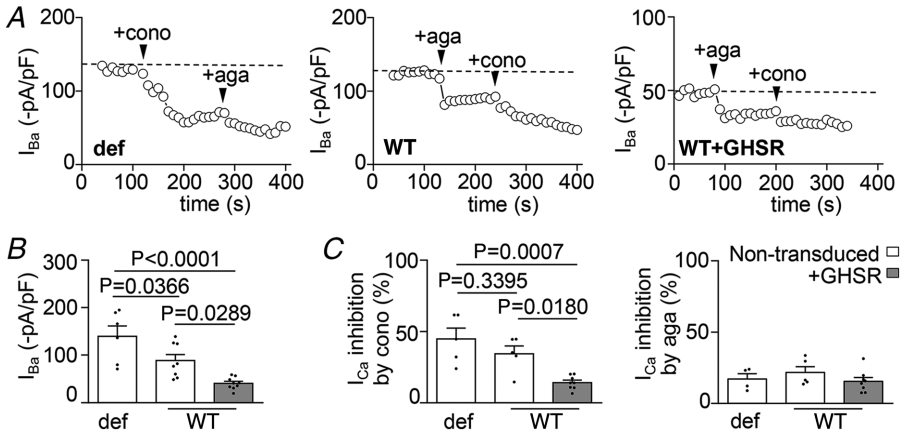

Figure 7. GHSR expression reduces the contribution of $Ca_V2.2$ to total Ca_V currents
I_{Ba} time courses of application of 1 μM ω-conotoxin-GVIA (+cono) and 0.2 μM ω-agatoxin-IVA (+aga) (*A*), averaged basal I_{Ba} levels (*B*) and average percentage of I_{Ba} inhibition (*C*) from >12 DIV GHSR-deficient (def), wild-type (WT) or wild-type transduced with lentiviral plasmids encoding GHSR (WT+GHSR) hippocampal neurons. Statistical significance evaluated by Kruskal–Wallis ANOVA and Dunn's *post hoc* test.

Discussion

Ghrelin-induced GHSR activity controls neuronal function through multiple mechanisms. Postsynaptic mechanisms have been widely studied: GHSR controls transcriptional levels, enhances firing rates through K_V7 channel inhibition, and increases postsynaptic receptor membrane density and spine synapse number (Diano *et al.* 2006; Shi *et al.* 2013; Ribeiro *et al.* 2014). Despite the fact that many GPCRs act on neuronal circuits by regulating neurotransmitter release (Catterall & Few, 2008), information about GHSR impact on neurotransmission at the presynaptic level remains scarce (Cowley *et al.* 2003; Ribeiro *et al.* 2014; Lopez Soto, 2015; Cabral *et al.* 2016). In this regard, we have found that GABA release from hypothalamic explants is reduced when mice are exposed to fasting conditions, in which GHSR mRNA levels are increased, indicating that GHSR constitutive activity reduces GABAergic tone in the hypothalamus (Lopez Soto, 2015). Here we show that GHSR constitutive activity impairs inhibitory neurotransmission in hippocampal neurons in culture. Our data support a presynaptic effect mediated mainly by $Ca_V2.2$ current impairment that specifically affects GABA release. This study and our previous work add information to the several reports assaying ghrelin's effect in neurons (Cowley *et al.* 2003; Moran & Gao, 2006; Sleeman & Spanswick, 2014; Cabral, *et al.* 2015; Hsu *et al.* 2016) contributing to the understanding of the physiology of this GPCR, which has the highest constitutive activity known (Holst & Schwartz, 2004; Mear *et al.* 2013).

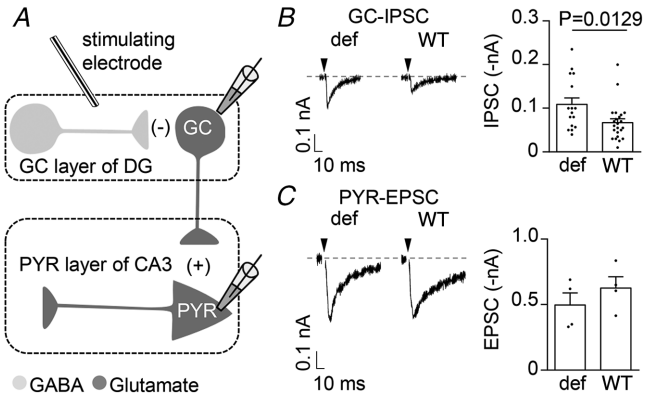

Figure 9. GHSR constitutive activity modulates synaptic transmission in hippocampal slices
A, diagram of the DG-CA3 circuit showing positions of extracellular stimulating and recording electrodes on the granule cell layer of the dentate gyrus (GC layer of DG) and pyramidal cell layer of CA3 (PYR layer of CA3). *B*, representative traces and average intra-granule cell layer IPSCs (GC-IPSCs) from wild-type (WT) and GHSR-deficient (def) mice. *C*, representative traces and average EPSCs from WT and GHSR-deficient (def) pyramidal cell layer (PYR-EPSCs) mice. Statistical significance evaluated by Mann–Whitney test.

Ca_V2 channels are fundamental for neurotransmission, as they allow calcium influx into the presynaptic terminal which triggers neurotransmitter exocytosis (Dunlap *et al.* 1995). Therefore, control of Ca_V2 activity is essential for modulating neuronal processes (Catterall & Few, 2008), relying on mechanisms such as changes in Ca_V membrane distribution or density (Davidova, 2014; Ferron, 2014; Nakamura *et al.* 2015; Schneider *et al.* 2015), or changes in Ca_V activation and deactivation kinetics (Burgoyne & Weiss, 2001; Catterall & Few, 2008). Many GPCRs modulate Ca_V2 activity. We have previously shown that ghrelin-induced GHSR activation acutely modulates $Ca_V2.1$ and $Ca_V2.2$ currents and neurotransmitter release. Moreover, we found that GHSR constitutive activity can also impair Ca_V currents in a chronic, $G_{i/o}$- and $Ca_V\beta$-subunit-dependent and voltage-independent manner (Lopez Soto, 2015; Mustafá *et al.* 2017). The impairment of Ca_V currents by GHSR constitutive activity is related to a decrease in Ca_V channel density at the plasma membrane together with a concomitant increase in Ca_V channel density at the endoplasmic reticulum and Golgi apparatus. Hence, we postulate that GHSR constitutive signalling reduces Ca_V channel forward trafficking resulting in a reduction in the Ca_V channel surface density and current. Since this chronic effect relies on GHSR sustained signalling, it would be independent from GHSR subcellular location. On the other hand, we expect that this effect would be more relevant during periods of active Ca_V2 channel trafficking such as synaptogenesis, development and adult neurogenesis (Bergami *et al.* 2015; Nakamura *et al.* 2015). In this context, we reasoned that primary neuronal culture was an adequate model to uncover the functional effect of GHSR constitutive activity at the presynaptic terminals. This model system is widely used to study the properties of synaptic transmission at the cellular level as it constitutes a preparation with a controlled environment which allows recapitulation of the properties of neuronal cells *in vivo* (Basarsky *et al.* 1994; Matteoli *et al.* 1995; Verderio *et al.* 1999). In this experimental setting we found that GHSR-deficient neurons displayed an augmented level of total Ca_V current, and overexpressing GHSR avoided the Ca_V current increase over days in culture (Fig. 1). As expected, this effect at the synaptic level only modulates Ca_V-dependent neurotransmission. We found no effect of GHSR expression on mEPSC and mIPSC or on GABA and glutamate released by application of hyperosmotic solution. Though we expected GHSR to be expressed on different neuronal subtypes (especially in conditions with receptor overexpression), GHSR constitutive activity specifically affects the Ca_V-dependent release of GABA (Fig. 2).

If Ca_V currents are reduced indiscriminately in hippocampal neurons by GHSR constitutive activity, why does this effect target GABA but not glutamate release?

We postulate that the preferential reliance of GABA release on $Ca_V2.2$ over $Ca_V2.1$ channels could underlie this selectivity. Indeed, it is expected that a reduction in the number of presynaptic calcium channels would have a greater effect on neurotransmitter release at synapses that depend mainly on $Ca_V2.2$ rather than on $Ca_V2.1$, since it has been demonstrated that $Ca_V2.1$ channels are more tightly coupled to synaptic vesicles and have a larger open probability than $Ca_V2.2$ channels at depolarizing potentials (Stanley, 2015). Furthermore, Cao and Tsien (2010) overexpressed both permeable and impermeable Ca_V versions in hippocampal neurons in culture and showed that $Ca_V2.1$ and $Ca_V2.2$ interact differentially with specific binding sites (slots) at the presynaptic terminals, thus regulating the relative contribution of each subtype to neurotransmitter release (Cao & Tsien, 2010). In particular, the number of presynaptic $Ca_V2.1$ channels present saturates a particular subgroup of slots, imposing a ceiling on the synaptic efficacy of these Ca_V channels. Thus, synapses that are mostly governed by $Ca_V2.2$ would be more sensitive to changes in the number of available channels compared to $Ca_V2.1$-enriched presynaptic terminals. In addition, excitatory and inhibitory synapses rely differentially on Ca_V2 subtypes. Previous studies support a differential reliance of GABA and glutamate release on $Ca_V2.2$ that could explain the specific effect of GHSR on GABA release that we describe. Several studies also show that upon excitatory synapse maturation there is a switch from $Ca_V2.2$ to $Ca_V2.1$ coupling to glutamate release (Scholz & Miller, 1995; Pravettoni *et al.* 2000; Cao & Tsien, 2010). In contrast, GABA release has been shown to depend to a higher extent on $Ca_V2.2$ calcium influx upon synapse maturation (Basarsky *et al.* 1994). Remarkably, overexpression of $Ca_V2.2$ in neuronal cultures does not result in an increase in glutamate release, although the Ca_V currents do increase (Cao & Tsien, 2010). Furthermore, we observed a larger contribution of $Ca_V2.2$ to the total current and found that GHSR reduces the relative $Ca_V2.2$ contribution to the total Ca_V currents in culture. This is in agreement with previous observations indicating that $Ca_V2.2$ make a greater contribution than $Ca_V2.1$ to the total Ca_V current in hippocampal neuronal cultures (Pravettoni *et al.* 2000).

In terms of our choice of experimental setting, we consider that primary culture is a suitable model to study a mechanism that interrupts Ca_V channel trafficking because it recapitulates axonal and dendritic growth as well as synapse formation in a controlled manner. Despite this, we are aware that exploring the impact of GHSR modulation in an intact hippocampal circuit is necessary for a better understanding of its physiological impact. In the hippocampal formation, the dentate gyrus is a key structure for different types of learning in humans, primates and rodents, given that it is the entry point to the trisynaptic glutamatergic circuit (Burgess, 2002; Leutgeb *et al.* 2005; Bakker *et al.* 2008). Acting as an information filter, the granule cells of the dentate gyrus display low intrinsic activity (Liu *et al.* 2012; Ramirez *et al.* 2013; Denny *et al.* 2014; Danielson *et al.* 2016), strongly controlled by local GABAergic innervation (Nitz & McNaughton, 2004; Sambandan *et al.* 2010; Pernía-Andrade & Jonas, 2014). In acute hippocampal slices, we found that GHSR-deficient mice display larger GC-IPSCs than WT mice (Fig. 9*B*), indicating that GHSR constitutive activity can regulate the hippocampal circuit at the dentate gyrus level. To confirm that GHSR fails to affect the excitatory output from the dentate gyrus to CA3, we recorded EPSCs in CA3 stimulating the granule cell layer (Fig. 9*C*). We found no differences between GHSR-deficient and WT slices. This suggests that GHSR in the dentate gyrus affects exclusively GABAergic transmission. The selective effect of GHSR constitutive activity towards $Ca_V2.2$-dependent GABAergic transmission could have important physiological implications for the hippocampal circuit. Further experiments should be conducted to address if the modulation of GC-IPSCs by GHSR can affect the hippocampal excitatory/inhibitory balance or short-term plasticity depending solely on GHSR expression levels (like the decrease in inhibitory PPR we observed in primary cultures, Fig. 3). Also, it would be interesting to investigate if this mechanism could support an already described presynaptic variant of long-term depression in the intact brain. This form of plasticity has been shown for other $G_{i/o}$ GPCRs that produce a sustained depression after application and washing of agonist (Kim & Kita, 2013). In the case of GHSR, the depression would originate from the sole expression of this receptor in a period of high Ca_V2 trafficking.

How could neurons tune the effect of this basally active GPCR? The answer could arise from different physiological situations. In the first place, it has been reported that GHSR expression in the hippocampus varies significantly within DIV (Lattuada *et al.* 2013; Ribeiro *et al.* 2014). The developmental profile of GHSR in hippocampal cultured neurons analysed by mRNA expression, western blots and immunofluorescence assays shows that GHSR expression levels are regulated during synapse development *in vitro*, exhibiting a significant increase from 7 to 21 DIV, in agreement with the timing in which we observed a strong increase in Ca_V currents in GHSR-deficient neurons. Also, differences in the organism's energy balance caused by fasting can induce changes in GHSR expression levels in the rodent hypothalamus (Kim *et al.* 2003; Fernandez *et al.* 2018). On the other hand, GHSR has been described as heterodimerizing with other GPCRs, and this association could modify the basal signalling of GHSR constitutive activity (Kern *et al.* 2015; Schellekens *et al.* 2015; Wellman & Abizaid, 2015). Finally, GHSR constitutive activity could be adjusted by

putative naturally occurring inverse agonists. We have previously shown that the chronic effect on Ca_V1 by another constitutively active GPCR, the melanocortin receptor type 4, can be prevented by its natural inverse agonist, agouti-related peptide (Agosti *et al.* 2017). Although a specific natural inverse agonist for GHSR has not been discovered to date, many efforts are being made to develop synthetic inverse agonist with potential pharmacological actions (Holst *et al.* 2006, 2007; Moulin *et al.* 2007; Damian *et al.* 2012; Els *et al.* 2012).

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Additional information

Competing interests

The authors declare no competing financial interests

Author contributions

All experiments presented in this work were performed in the Electrophysiology Laboratory of IMBICE. J.R. directed the conception and experimental design and contributed to data interpretation. S.S.R. amplified and purified the clones for the lentiviral vector assembly. V.M.D. performed data acquisition, analysis and interpretation. J.R. and V.M.D. prepared, wrote and revised the manuscript. All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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