ACS Chemical Neuroscience

Subscriber access provided by UNIV OF WESTERN ONTARIO

Article

Dopamine receptor type 2 (D2R) and ghrelin receptor (GHSR) co-expression alters Ca2.2 modulation by G protein signaling cascades.

Santiago Cordisco Gonzalez, Emilio Román Mustafá, Silvia S. Rodriguez, Mario Perello, and Jesica Raingo

ACS Chem. Neurosci., **Just Accepted Manuscript** • DOI: 10.1021/acschemneuro.9b00426 • Publication Date (Web): 06 Dec 2019

Downloaded from pubs.acs.org on December 7, 2019

Just Accepted

"Just Accepted" manuscripts have been peer-reviewed and accepted for publication. They are posted online prior to technical editing, formatting for publication and author proofing. The American Chemical Society provides "Just Accepted" as a service to the research community to expedite the dissemination of scientific material as soon as possible after acceptance. "Just Accepted" manuscripts appear in full in PDF format accompanied by an HTML abstract. "Just Accepted" manuscripts have been fully peer reviewed, but should not be considered the official version of record. They are citable by the Digital Object Identifier (DOI®). "Just Accepted" is an optional service offered to authors. Therefore, the "Just Accepted" Web site may not include all articles that will be published in the journal. After a manuscript is technically edited and formatted, it will be removed from the "Just Accepted" Web site and published as an ASAP article. Note that technical editing may introduce minor changes to the manuscript text and/or graphics which could affect content, and all legal disclaimers and ethical guidelines that apply to the journal pertain. ACS cannot be held responsible for errors or consequences arising from the use of information contained in these "Just Accepted" manuscripts.

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036
Published by American Chemical Society. Copyright © American Chemical Society. However, no copyright claim is made to original U.S. Government works, or works produced by employees of any Commonwealth realm Crown government in the course of their duties.

Title

Dopamine receptor type 2 (D2R) and ghrelin receptor (GHSR) co-expression alters $Ca_V2.2$ modulation by G protein signaling cascades.

Santiago Cordisco Gonzalez[1], Emilio Román Mustafá[1], Silvia S. Rodriguez[1], Mario Perello[2] and Jesica Raingo[1*]

[1]Laboratory of Electrophysiology and [2]Laboratory of Neurophysiology of the Multidisciplinary Institute of Cell Biology (IMBICE), B1904CMA La Plata, Buenos Aires, Argentina.

*Corresponding author jraingo@gmail.com jraingo@imbice.gov.ar

Abstract

Voltage-gated calcium channels type 2.2 ($Ca_V2.2$) are activated by action potentials at presynaptic terminals, and their calcium current induces neurotransmitter release. In this context, regulating $Ca_V2.2$ is critical, and one of the most important mechanisms for doing so is through is G protein-coupled receptor (GPCR) activity. Two such GPCRs are the ghrelin (GHSR) and the dopamine type 2 (D2R) receptors. We previously demonstrated that constitutive GHSR activity reduces $Ca_V2.2$ forward trafficking and that ghrelin-induced GHSR activity inhibits $Ca_V2.2$ currents. On the other hand, dopamine-induced D2R activity also inhibits $Ca_V2.2$ currents. It has been recently shown that D2R and GHSR form heteromers in hypothalamic neurons. This interaction profoundly changes the signaling cascades activated by dopamine and is necessary for dopamine-dependent anorexia. Here we explored how D2R-GHSR co-expression in HEK293T cells modulates the effect that each GPCR has on $Ca_V2.2$. We found that D2R-GHSR co-expression reduces the inhibition of $Ca_V2.2$ currents by agonist-induced D2R activation and added a new source of basal $Ca_V2.2$ current inhibition to the one produced by GHSR solely expression. We investigated the signaling cascades implicated and found that constitutive GHSR activity, G_q protein and Gβγ subunit play a critical role in these altered effects. Moreover, we found that the effect of D2R agonist on native calcium currents in hypothalamic neurons is reduced when both D2R and GHSR are over-expressed. In summary, our results allow us to propose a novel mechanism for controlling $Ca_V2.2$ currents involving the co-expression of two physiologically relevant GPCRs.

Keywords

Dopamine, ghrelin, hypothalamus, calcium channels, G protein, heteromers.

Introduction

G protein-coupled receptors (GPCRs) are important for controlling neuronal functions [1-3]. The cellular effects of a given GPCR vary based on neuronal type and environmental conditions. Thus, defining the main G protein subtype that is coupled and the downstream intracellular pathways that are activated is insufficient to explain the diverse functions of a given GPCR. In this context, understanding non-canonical properties of

GPCRs, which critically change their global effect, can help to illuminate complex GPCR behavior. Among these non-classical properties are constitutively active states [4-6], promiscuous G protein activation by different natural and pharmacological agonists [7-9], and GPCR homo- and heteromerization [10-12].

Several GPCR heteromers have been described with the advance of biochemical and imaging techniques such as FRET (Förster Resonance Energy Transfer) and BRET (bioluminescence resonance energy transfer) [13]. The GPCRs more prone to form heteromers include the dopamine receptors and the ghrelin receptor (GHSR) [14, 15]. A physiologically relevant example of this non-canonical GPCR behavior is the dopamine type 2 receptor (D2R) and GHSR heteromerization in hypothalamic neurons, where their interaction is required for the anorexigenic effect of D2R activation by agonists [12]. When both receptors are co-expressed on the same neuron, the dopamine-induced D2R signaling pathway activation switches from adenylate cyclase (AC) inhibition to calcium mobilization from internal stores in a Gβγ- and phospholipase C (PLC)-dependent manner [12, 16]. An important question that arises from these observations is: does D2R-GHSR interaction impact other neuronal targets?

D2R and GHSR signaling modulate presynaptic Ca_V2 channels, which are key effectors for their actions. In particular, the activation of these two GPCRs independently inhibits presynaptic $Ca_V2.2$ subtype, affecting neurotransmitter release [5, 17-19]. Dopamine-induced D2R activation reduces $Ca_V2.2$ currents in a $G_{i/o}$ protein dependent manner, while ghrelin-induced GHSR activation acts through its coupling to G_q protein [5, 20]. On the other hand, we have previously demonstrated that constitutive GHSR activity, through the $G_{i/o}$ signaling pathway, affects $Ca_V2.2$ trafficking to the plasma membrane and consequently reduces $Ca_V2.2$ calcium currents [21]. Here, we aimed to evaluate if D2R and GHSR co-expression differentially impacts on $Ca_V2.2$ current modulation.

Results and discussion

First we explored the effect of consecutive applications of D2R and GHSR agonists (quinpirole 10 μM and ghrelin 0.5 μM) on native Ca_V currents in hypothalamic cultured neurons, where D2R-GHSR co-expression has been previously reported [12]. For both agonists, we observed a range of inhibition on Ca_V currents, as expected for a native system [5, 17]. Specifically, we noticed that neurons with a large inhibitory response to quinpirole exhibited a mild inhibitory response to ghrelin (see example a in figure 1). Conversely, neurons that were less sensitive to quinpirole displayed a large response to ghrelin (see example b in figure 1). Quinpirole and ghrelin effects were reversible upon washout as Ca_V currents were fully recovered (data not shown) were reversible upon washout as Cav currents. We estimated the Spearman's correlation coefficient between the degree of quinpirole- and ghrelin-induced inhibitions and found a significant inverse correlation (figure 1). Assuming that larger inhibitory effects are mainly due to higher receptor expression levels and based on the correlation between the effects of ghrelin and quinpirole, we hypothesize that changes in Ca_V current inhibition due to D2R and/or GHSR agonists is a new functional output of D2R-GHSR heteromerization in hypothalamic neurons.

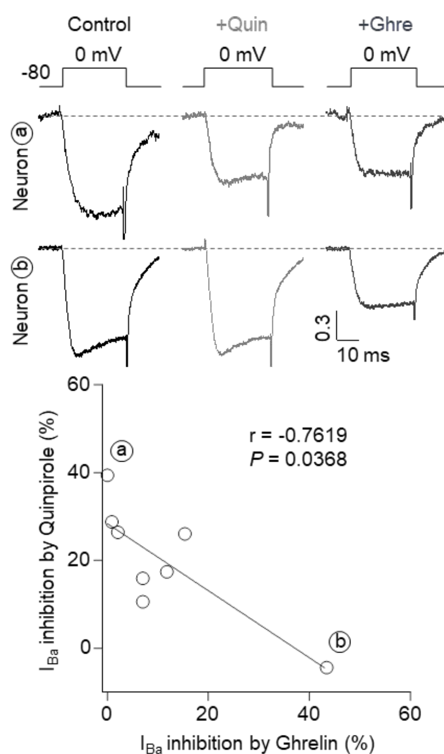


Figure 1. Barium current inhibition by quinpirole and ghrelin are inversely correlated in hypothalamic neurons. *Examples of barium current (I_{Ba}) traces before and after consecutive applications of quinpirole (10 µM, +Quin) and ghrelin (0.5 µM, +Ghre) from 2 cultured mouse embryonic hypothalamic neurons named a and b. Correlation between the percent inhibition of I_{Ba} by quinpirole versus ghrelin was calculated (Spearman`s correlation coefficient, r, and P value indicated). The best lineal fit line is also displayed (r^2=0.6615, slope=-0.7633 P=0.0141 versus zero).*

We next evaluated this correlation in a more controlled manner by over-expressing D2R and GHSR in hypothalamic cultured neurons. We used a lentiviral system with a high efficiency of transduction, resulting in homogeneous expression of each GPCR within the neuronal population, and evaluated the inhibitory effect of quinpirole and ghrelin in neurons over-expressing D2R, GHSR, or both GPCRs. Under these experimental conditions, we expected that over-expressing one receptor, but not the other, would increase the fraction of over-expressed receptor detached from the other. Conversely, we expected that the simultaneous over-expression of both receptors would increase the fraction of D2R-GHSR heteromers. As we show in figure 2A, the inhibitory effect of ghrelin on Ca_V currents was larger in neurons over-expressing GHSR than in neurons over-expressing D2R. Moreover, the effect of ghrelin in neurons co-expressing both GPCRs was not significantly different from neurons expressing GHSR alone. For quinpirole, we observed a larger effect in neurons over-expressing D2R alone compared to neurons expressing GHSR only (figure 2B). Importantly, we found a significant reduction in the inhibitory effect of quinpirole in neurons co-expressing D2R and GHSR. This result indicates that D2R-GHSR co-expression modulates quinpirole-mediated inhibition of Ca_V currents, while ghrelin-mediated inhibition remains unaffected. We next plotted the percent inhibition by quinpirole and ghrelin for the sets of neurons in which we were able to evaluate the effect of both agonists, as we did previously for uninfected neurons (figure 1). Figure 2C displays a lack of correlation for each group and reveals that the data can be segregated into two groups: neurons over-expressing D2R (squares), which are highly sensitive to quinpirole and lowly sensitive to ghrelin; and neurons over-expressing either GHSR or both GPCRs (triangles and circles, respectively), which have similar sensitivity to quinpirole and ghrelin. These results support the idea that D2R-GHSR co-expression alters the agonist-induced D2R signaling pathway that inhibits

native Ca_V currents, while agonist-induced GHSR inhibition of Ca_V currents remains unaffected. This is in agreement with previous data from Kern *et al.* showing that the co-expression of D2R and GHSR modifies quinpirole signaling cascade in a hypothalamic neuronal cell line [12].

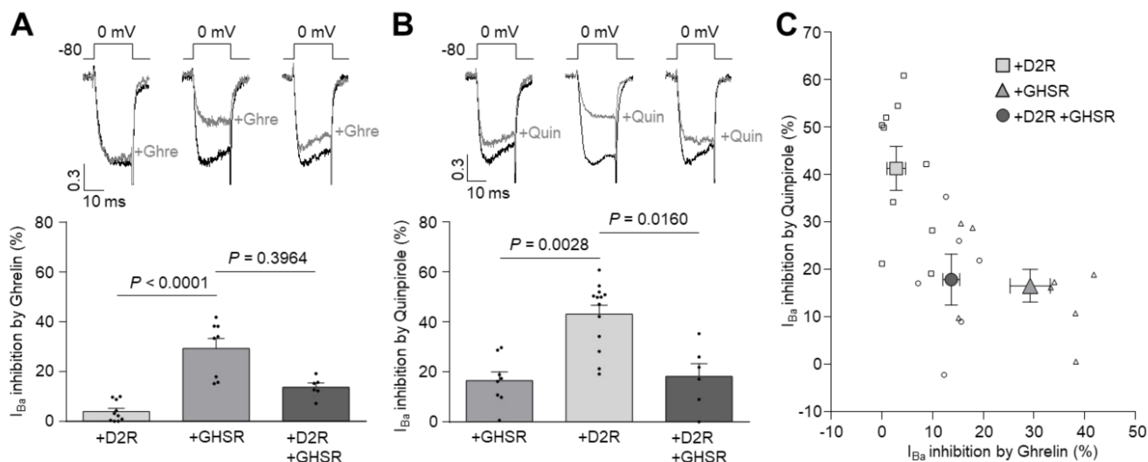


Figure 2. Simultaneous over-expression of D2R and GHSR in hypothalamic neurons reduces the inhibitory effect of quinpirole on barium currents. A. *Representative traces (top) of normalized barium current (I_{Ba}) from cultured mouse embryonic hypothalamic neurons transduced by lentivirus with D2R (+D2R, n=10), GHSR (+GHSR, n=8), or both receptors (+D2R +GHSR, n=6), before and after ghrelin application (0.5 µM, +Ghre). Bars (bottom) represent average percent inhibition of I_{Ba} by ghrelin. Statistical significance was evaluated by Kruskal-Wallis and Dunn's post-test.* ***B.*** *Representative traces (top) of I_{Ba} from cultured mouse embryonic hypothalamic neurons transduced by lentivirus with GHSR (+GHSR, n=8), D2R (+D2R, n=13), or both receptors (+D2R +GHSR, n=6) before and after quinpirole (10 µM, +Quin) application. Bars (bottom) represent average percent inhibition of I_{Ba} by quinpirole. Statistical significance was evaluated by Kruskal-Wallis and Dunn's post-test.* ***C.*** *Scatter plots of the percent inhibition of I_{Ba} by quinpirole versus ghrelin, calculated from hypothalamic neurons transduced by lentivirus with D2R (squares, n=10), GHSR (triangles, n=8), or both receptors (circles, n=6). Empty small symbols represent individual values. Correlation between the percentage of I_{Ba} inhibition by quinpirole and ghrelin was calculated in each data group and the Spearman`s correlation coefficient, r, and P value were r=-0.1945 and P=0.5755 for +D2R, r=-0.2143 and P=0.6191 for +GHSR, and r=0.2000 and 0.7139 for +D2R +GHSR not reaching statistical significance in any case. Colored big symbols represent the average values ± SEM for ghrelin and quinpirole effects.*

To gain insights of the mechanisms underlying the effects of D2R-GHSR co-expression on Ca_V currents, we ran experiments in HEK293T cells expressing the Ca_V2.2 channel. We chose this Ca_V subtype because it is the calcium channel most sensitive to GPCR activity and is the best studied in terms of its modulation by GHSR and D2R [5, 18-22]. We assayed the GHSR/D2R interaction in our experimental setting by FRET between GFP (donor) and mCherry (acceptor) by flow cytometry stimulating with a 488 nm laser. The experiments were done in HEK293T cells co-expressing GHSR-mCherry, soluble GFP and GHSR-

mCherry (negative control), GHSR-GFP and GHSR-mCherry (positive control see [23]) and D2R-GFP and GHSR-mCherry. In supplementary figure S1A we showed that in the condition co-expressing D2R-GFP and GHSR-mCherry the size of cell population with the highest red fluorescent signal is significantly larger than the negative control condition and not different from the positive control condition. Moreover we run an internalization assay to evidence the interaction between GHSR and D2R in our system similar to the report by Evans *et al.* [24] and others [25, 26]. In this case, we took advantage of the fact that GHSR displays ghrelin-mediated internalization and explore if D2R co-internalize with GHSR. As we show in the supplementary figure S1B we recapitulated the ghrelin mediated internalization of GHSR by ghrelin as we have shown before [27]. More important, exposing cells to 0.5 μM ghrelin reduces D2R in the cell surface and increases it in internal compartments only when GHSR and D2R are co-expressed. Thus we confirmed that GHSR and D2R interact in our experimental system *in vitro*. Next we performed patch clamp experiments in cells co-transfected either D2R, GHSR, or both D2R and GHSR, with each receptor at a 0.1 molar ratio to the $Ca_V2.2$ cDNA. We first tested the percentage of inhibition of saturating doses of ghrelin (0.5 μM) [5, 28] on whole-cell calcium currents and found no differences between cells expressing GHSR or both GPCRs (figure 3A). This observation agrees with our previous data showing the effects of ghrelin on neurons over-expressing GHSR as compared to neurons expressing both GPCRs. We next assayed the effect of 10 μM dopamine and found that the percentage of current inhibition is reduced by co-expressing GHSR (figure 3B). In order to test whether constitutive GHSR activity is involved in the reduction of the dopamine-mediated inhibition of $Ca_V2.2$ currents, we tested the effect of 1 μM SPA (substance P analog), a GHSR ligand that stabilizes the receptor in an inactive state [29] and also uncouples G_q from the receptor [30]. We pre-incubated cells co-expressing D2R and GHSR for 20 hours with SPA and found that this maneuver restored the inhibitory effect of dopamine to control levels (figure 3B). Moreover, we tested the effects of dopamine in cells co-expressing D2R and a mutated version of GHSR (GHSRA204E), [5, 31]) that couples to G_q [30] but lacks constitutive activity. We found that the level of $Ca_V2.2$ current inhibition by dopamine was equal to that observed in D2R-expressing cells. These results suggest that constitutive GHSR activity is necessary for the reduced effect of D2R agonist on $Ca_V2.2$ currents in presence of GHSR. However we cannot rule out the possibility that the rescue of the full inhibitory effect of dopamine by SPA and the GHSR mutant is due to other causes: the inability of G_q to signal [30], and/or the lower degree of D2R-GHSR heteromer formation. In this regard, Kern *et al*. (2012) have proposed that several GHSR single mutants and SPA incubation modify the GHSR conformational state, preventing its interaction with D2R [12].

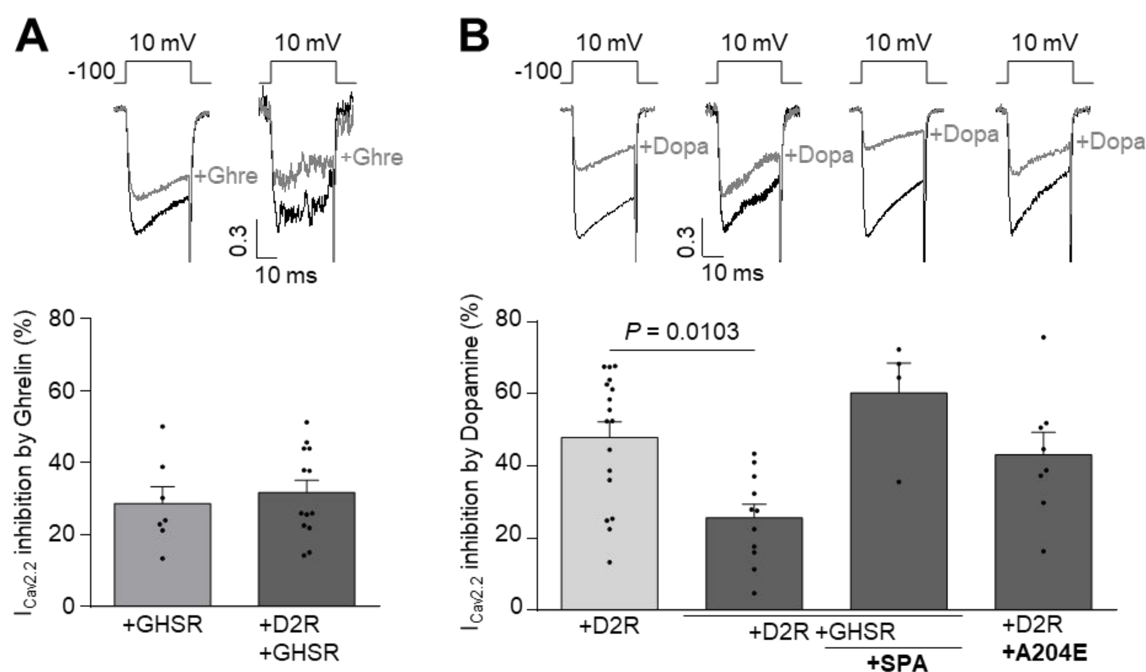


Figure 3.** **Ca_V2.2 current inhibition by dopamine-mediated D2R activation is reduced in presence of GHSR in a GHSR constitutive activity-dependent manner. A. *Representative traces (top) of normalized Ca_V2.2 calcium currents ($I_{CaV2.2}$) from HEK293T cells co-transfected with Ca_V2.2 and either GHSR (+GHSR, n=7), or GHSR and D2R (+D2R +GHSR, n=13), before and after ghrelin application (0.5 μM, +Ghre). Bars (bottom) represent average percent inhibition of $I_{CaV2.2}$. Statistical significance evaluated by unpaired t-test, P=0.6049.* ***B.*** *Representative traces (top) of normalized $I_{CaV2.2}$ from HEK293T cells co-transfected with Ca_V2.2 and either D2R (+D2R, n=17), or D2R and GHSR pre-incubated (+D2R +GHSR +SPA, n=4) or not (+D2R +GHSR, n=11) with SPA (1 μM), or D2R and GHSRA204E (+D2R +A204E, n=8), before and after dopamine application (10 μM, +Dopa). Statistical significance evaluated by Kruskal-Wallis and Dunn's post-test (versus +D2R).*

In order to determine if the weaker effect of dopamine in presence of GHSR is due to a reduced affinity of dopamine to D2R and/or to a change in dopamine efficacy, we performed concentration-response curves for the dopamine effect on HEK293T transfected with Ca_V2.2 and either D2R alone or both D2R and GHSR. We found that the co-expression of GHSR significantly changes the maximum percent current inhibition (figure 4).The EC_{50} values were in the range of the K_D reported for dopamine binding to D2R [32]. The change in the efficacy of dopamine to inhibit Ca_V2.2 without a change in its affinity to bind the receptor suggests that either dopamine acts as a partial agonist when both receptors are expressed or the intracellular pathways available to D2R are changed in the presence of GHSR and consequently dopamine would behave as a biased agonist [33]. Interestingly, we found that the efficacy of dopamine to inhibit Ca_V2.2 currents was restored to control values when on HEK293T transfected with Ca_V2.2 and both D2R and GHSR were pre-incubated with SPA. Thus, this observation suggest that the conformational state of GHSR,

either per se or due to ability to activate signaling cascades, is important to impair dopamine-mediated inhibition of $Ca_V2.2$ currents via D2R.

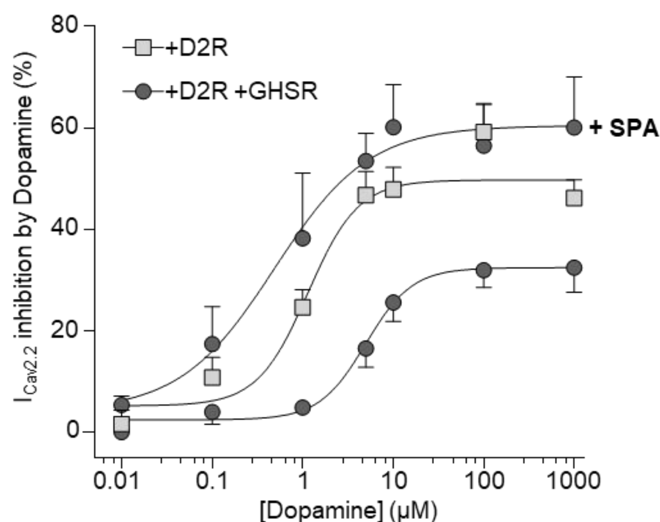


Figure 4. The efficacy of dopamine to inhibit $Ca_V2.2$ current is reduced when GHSR is co-expressed. *Dopamine concentration (0.01–1000 µM)–response curve of $Ca_V2.2$ calcium current inhibition in HEK293T cells co-transfected with $Ca_V2.2$ and either D2R (squares), or D2R and GHSR (circles), pre-incubated or not with SPA (1 µM, +SPA). Symbols represent the average percent inhibition of calcium current and lines represent the fitted Hill equation. The n values for each condition are: 89 for +D2R, 48 for +D2R +GHSR and 28 for +D2R +GHSR +SPA (ranging from 3 to 25 for each data set).The maximum inhibition for the D2R and GHSR condition (99% confidence interval= 24.65 to 41.22) is significantly different from the one in the D2R (99% confidence interval= 44.30 to 53.16) and in the D2R and GHSR in presence of SPA (99% confidence Interval=42.26 to 72.88) conditions.*

D2R couples to $G_{i/o}$, and the main reported downstream pathways activated by agonist are: 1) inhibition of adenylate cyclase by $G\alpha_{i/o}$, 2) direct activation of inward rectifier potassium channels (GIRK) [34], and 3) direct inhibition of native $Ca_V2.2$ calcium currents by Gβγ [17]. Dopamine-activated D2R also inhibits $Ca_V2.2$ through an additional Gβγ-independent mechanism observed in a heterologous system [20]. Moreover, D2R also activates PLC in a Gβγ-dependent manner [35]. Kern *et al.* (2012) have reported that the D2R and GHSR interaction perturbs the intracellular pathway downstream of $G_{i/o}$ activation by dopamine, increasing the activation of PLC and inducing the release of calcium from intracellular compartments [12]. This effect is mediated by G_q coupled to GHSR [23]. Thus, we hypothesized that G_q and Gβγ could be involved in the reduction of dopamine-mediated inhibition of $Ca_V2.2$ when D2R and GHSR are co-expressed. We therefore tested the effect of dopamine in cells co-expressing D2R and GHSR, with or without the addition of a G_q dominant negative protein (G_q DN) that occludes the downstream effect of G_q without altering GPCR conformation [36]. We found that the effect of dopamine on $Ca_V2.2$ currents remains unchanged in cells expressing only D2R and G_q DN. In contrast, we observed that

the degree of $Ca_V2.2$ inhibition by dopamine was restored to control value (expressing D2R) in cells co-expressing D2R, GHSR and G_q DN (figure 5A). Moreover, in order to address the specificity of GHSR effect on D2R signaling we used an irrelevant G_q protein coupled receptor. As we show in figure 5B replacing GHSR by the angiotensin II type 1 receptor (AT1R) failed to modify the dopamine mediated inhibition of $Ca_V2.2$ currents. Since it has been shown before that AT1R activation by angiotensin II inhibits Ca_V currents [37] here we also tested the functionality of AT1R and found that angiotensin II 1 µM significantly reduces the $Ca_V2.2$ current in cells expressing AT1R and $Ca_V2.2$ (31.53±6.94%, n=3 cells, P=0.0451, Student's t-test versus zero). In summary our results suggest that GHSR-mediated activation of G_q is required for the inhibitory effect of GHSR co-expression on the effects of dopamine-evoked D2R activity that target $Ca_V2.2$.

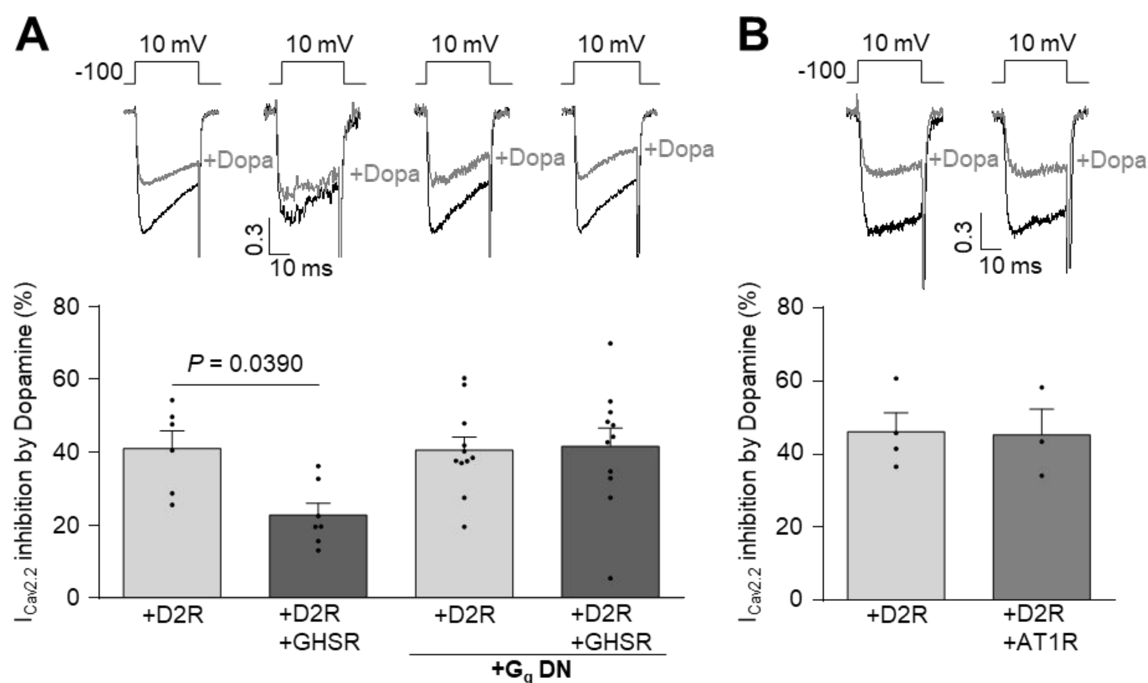


Figure 5. The reduced inhibition of $Ca_V2.2$ current by dopamine when GHSR is co-expressed depends on G_q protein. A. *Representative traces (top) of normalized $Ca_V2.2$ calcium current ($I_{CaV2.2}$) from HEK293T cells co-transfected with $Ca_V2.2$, D2R, and either pcDNA3.1(+) (+D2R, n=6), G_q DN (+D2R +G_q DN, n=11), GHSR (+D2R +GHSR, n=7) or GHSR and G_q DN (+D2R +GHSR +G_q DN, n=11), before and after dopamine application (10 µM, +Dopa). Bars (bottom) represent the average percent inhibition of $I_{CaV2.2}$. Statistical significance was evaluated by Kruskal-Wallis and Dunn's post-test (versus +D2R).* ***B.*** *Representative traces (top) of normalized $Ca_V2.2$ calcium current ($I_{CaV2.2}$) from HEK293T cells co-transfected with $Ca_V2.2$ and D2R (+D2R, n=4), or with D2R and AT1R (+D2R +AT1R, n=3) before and after dopamine application (10 µM, +Dopa). Bars (bottom) represent average percent inhibition of $I_{CaV2.2}$. Statistical significance evaluated by Student´s t-test.*

Next we assayed the involvement of Gβγ subunits to the reduced effect of dopamine when D2R and GHSR are co-expressed. First, we tested the effect of dopamine on $Ca_V2.2$ currents in HEK293T cells co-expressing D2R and MAS-GRK2-ct, a Gβγ buffer peptide [38, 39], and found that buffering Gβγ reduced the effect of dopamine in D2R expressing cells [20]. Interestingly, the low level of dopamine-mediated $Ca_V2.2$ inhibition in cells co-expressing D2R and GHSR remained unchanged when MAS-GRK2-ct was also co-expressed (figure 6). Taken together, our results indicate that the Gβγ role in dopamine mediated inhibition of $Ca_V2.2$ is lost when GHSR is co-expressed. Previous studies have postulated that activation of one GPCR can transfer its Gβγ subunit to the Gα subunit coupled to other GPCR, thereby reducing the availability of free Gβγ to exert downstream effects [40]. On the other hand Kern *et al.* [12] have shown that D2R and GHSR co-expression leads to a dopamine-induced PLC activation via Gβγ. A putative explanation to conceal this report with our current data is to postulate that basally active GHSR takes Gβγ subunits from $G_{i/o}$ coupled to D2R to activate PLC. In this scenario is possible to think that the amount of Gβγ available to bind $Ca_V2.2$ would be reduced. This idea is in agreement with other reports demonstrating a close interaction between $G_{i/o}$ and G_q when D2R and GHSR are co-expressed [23]. Thus, we hypothesize that constitutive GHSR activity could reduce the availability of free Gβγ to bind $Ca_V2.2$ and consequently impair dopamine-mediated D2R signaling pathways. In summary, the mechanisms involved in GHSR and D2R interaction seem to be very complex and may imply multiple pathways that differentially impact on other cellular structures different from $Ca_V2.2$.

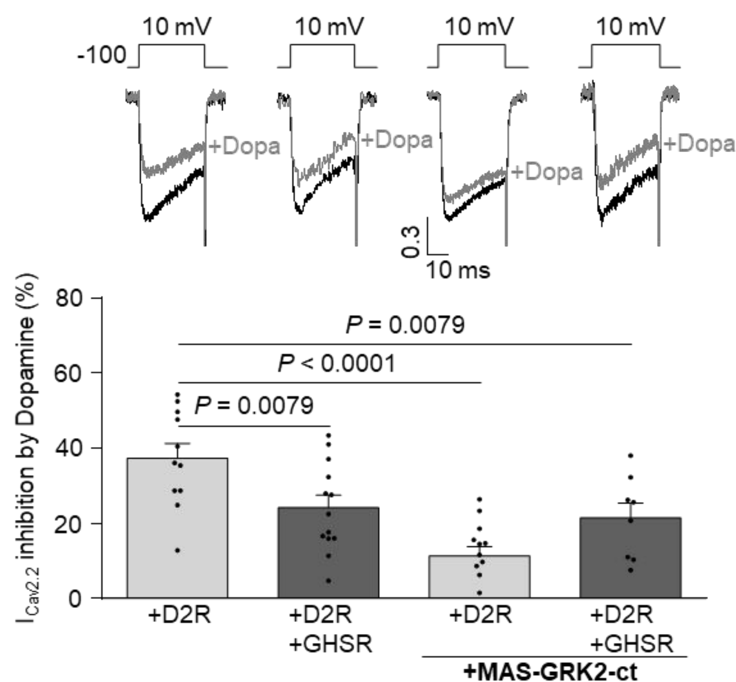


Figure 6. The reduced inhibition of $Ca_V2.2$ current by dopamine when GHSR is co-expressed involves the loss of Gβγ-mediated inhibition of $Ca_V2.2$. *Representative traces (top) of normalized $I_{CaV2.2}$ from HEK293T cells co-transfected with $Ca_V2.2$, D2R, and either pcDNA3.1(+) (+D2R, n=11), MAS-GRK2-ct (+D2R +MAS-GRK2-ct, n=13), GHSR (+D2R +GHSR, n=13) or GHSR and MAS-GRK2-ct (+D2R +GHSR +MAS-GRK2-ct, n=8)*

before and after dopamine application (10 μM, +Dopa). Bars (bottom) represent the average percent of $I_{CaV2.2}$ inhibition. Statistical significance was evaluated by Kruskal-Wallis and Dunn's post-test (versus +D2R).

In order to decipher how D2R and GHSR co-expression affects the dopamine- and ghrelin-evoked inhibition of $Ca_V2.2$ we have normalized the size of currents and compared the percent inhibition for each condition. However, we noticed that the basal $Ca_V2.2$ current densities in pre-normalized data were consistently reduced in cells co-expressing both receptors. Thus, we decided to investigate whether the co-expression of D2R and GHSR affects basal $Ca_V2.2$ currents. First we run control experiments displayed in supplementary figure S2 to confirm that manipulating the amount of cDNA for each GPCR plasmid correlates with GPCR plasma membrane expression. We used GFP- and mOrange-tagged versions of D2R and GHSR to determine fluorescent signal as a measurement of protein expression. Once we confirmed this positive correlation we compared the basal current levels of cells transfected with $Ca_V2.2$ alone (as a control) to cells transfected with D2R, GHSR, or D2R plus GHSR, at the following two different GPCR:$Ca_V2.2$ molar ratios: 0.1, as we used in all our previous experiments, and 0.5. We assayed 0.5 molar ratio because it is the threshold for basal $Ca_V2.2$ current reduction by GHSR constitutive activity as we described in our previous report [5]. We found a statistically significant reduction in basal $Ca_V2.2$ current only in the conditions co-expressing D2R and GHSR (figure 7). In the case of cells co-expressing D2R and GHSR in a 0.5 molar ratio, we found undetectable current levels, indicating more severe effects on $Ca_V2.2$ currents at higher GPCR:$Ca_V2.2$ molar ratio. Increasing the cDNA amount for D2R or GHSR alone did not produce statistically significant changes in the current levels. However, in accordance with our aforementioned study [5], we found that GHSR expression has a tendency to reduce basal $Ca_V2.2$ currents. D2R expression did not show any clear tendency in changing $Ca_V2.2$ current levels, in agreement with the non-conclusive data regarding D2R constitutive activity [41], though in the 0.1 D2R:$Ca_V2.2$ molar ratio condition, we did observe a slight increase in $Ca_V2.2$ current levels. This tendency of D2R expression to increase current levels at this molar ratio could be related to a previous report showing that co-expression of D2R increases $Ca_V2.2$ surface expression levels [20].

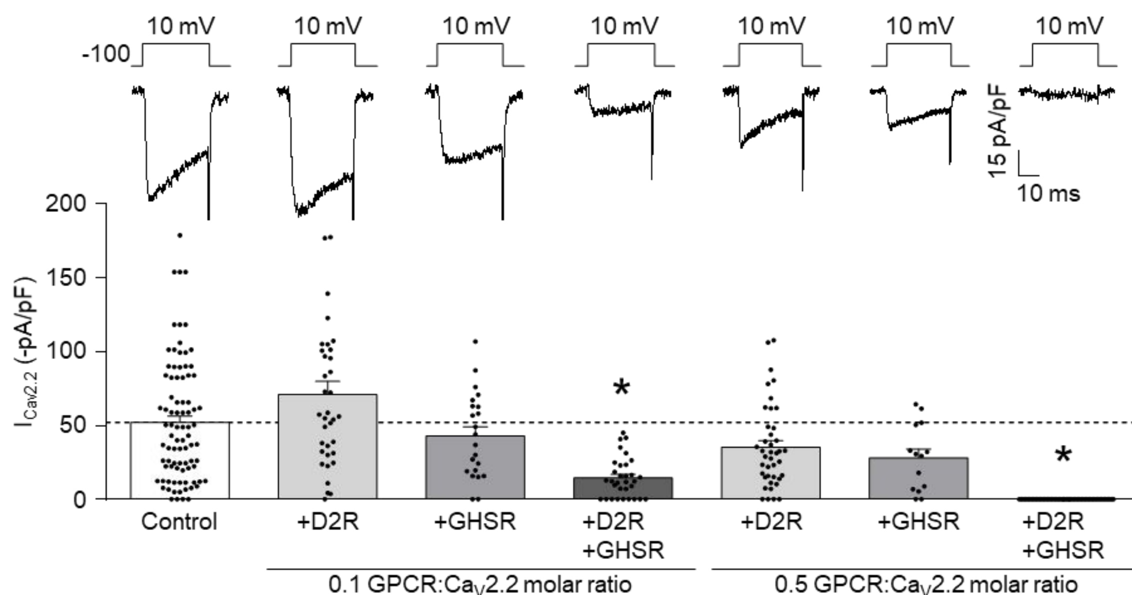


Figure 7. $Ca_V2.2$ basal current is reduced by GHSR and D2R co-expression. *Representative traces (top) of $Ca_V2.2$ calcium current ($I_{CaV2.2}$) from HEK293T cells co-transfected with $Ca_V2.2$ alone (Control, n=86), D2R, GHSR, or D2R plus GHSR in a 0.1 (+D2R, n=36, +GHSR, n=22, +D2R +GHSR, n=32) or 0.5 GPCR:$Ca_V2.2$ molar ratio (+D2R, n=41, +GHSR, n=14, +D2R +GHSR, n =53). Bars (bottom) represent the average $Ca_V2.2$ calcium current levels for each condition. Statistical significance was evaluated by Kruskal-Wallis and Dunn's post-test (versus Control, * P<0.0001).*

Next we explored the possibility that the reduction in basal current density in presence of both GPCRs could be due to a tonic Gβγ mediated inhibition. We performed experiments using the same protocol described by Evans *et al.* [24] to assess the facilitation of current due to pre-pulse mediated release of Gβγ tonic binding to the $Ca_V2.2$. In control conditions (in absence of GPCRs) we found a reduction of amplitude of $Ca_V2.2$ calcium current after the pre-pulse (I_2). We consider this effect mediated by an incomplete recovery from current inactivation. In cells co-expressing GHSR and $Ca_V2.2$ we found I_2/I_1 values similar to control indicating lack of Gβγ tonic inhibition in this condition. On the other hand, when we applied this protocol to cells co-expressing $Ca_V2.2$ and D2R the I_2/I_1 is significantly larger than control. Moreover, the I_2/I_1 ratio obtained in cells co-expressing both GPCRs was similar to control conditions or in presence of only GHSR with $Ca_V2.2$. These data indicate that there is a tonic effect of D2R on $Ca_V2.2$ currents that can be release by a strong depolarizing pre-pulse (figure 8A). To confirm the involvement of Gβγ in the basal current facilitation we repeated the experiments co-expressing the MAS-GRK2-ct, the Gβγ buffer peptide. This maneuver occluded the pre-pulse mediated current facilitation by D2R co-expression (figure 8B). This tonic inhibition of $Ca_V2.2$ currents by D2R co-expression may explain why we failed to observed the current density increase expected due to the reported D2R mediated rise of channel protein in the surface [5, 20]. More important, considering that Gβγ mediated inhibition of $Ca_V2.2$ by dopamine is lacking in presence of GHSR we could postulate that the

loss of tonic inhibition by D2R when co-expressing GHSR could be explained by Gβγ sequestration by basally active G_q/GHSR complexes.

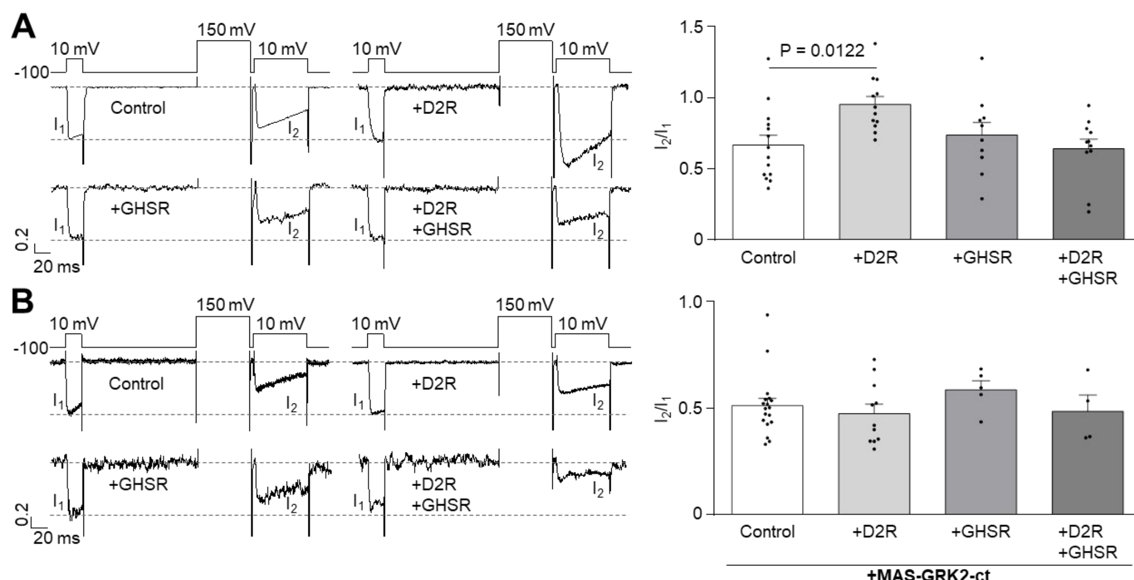
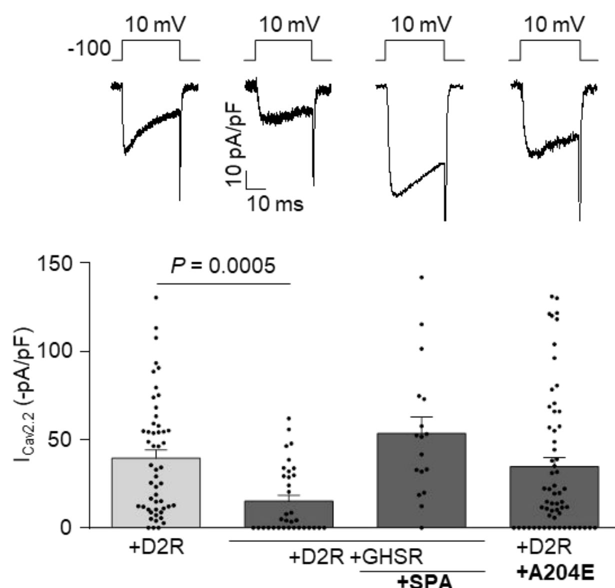


Figure 8. D2R-mediated Gβγ tonic inhibition of Ca_V2.2 is lost by GHSR co-expression. ***A***. *Representative traces (left) of Ca_V2.2 calcium current ($I_{CaV2.2}$) without (I_1) and with (I_2) a pre-pulse at +150 mV from HEK293T cells co-transfected with Ca_V2.2 alone (Control, n=14), D2R (+D2R, n=12), GHSR (+GHSR, n=10), or D2R plus GHSR (+D2R +GHSR, n=11) in a 0.1 GPCR:Ca_V2.2 molar ratio. Bars (right) represent the ratio between the peak current with and without pre-pulse (I_2/I_1) for each condition. Statistical significance was evaluated by ANOVA (versus Control).* ***B.*** *Representative traces (left) of Ca_V2.2 calcium current ($I_{CaV2.2}$) without (I_1) and with (I_2) a pre-pulse at +150 mV from HEK293T cells co-transfected with Ca_V2.2 alone (Control, n=18), D2R (+D2R, n=11), GHSR (+GHSR, n=5), or D2R plus GHSR (+D2R +GHSR, n=4) and MAS-GRK2-ct in a 0.1 GPCR:Ca_V2.2 molar ratio. Bars (right) represent the ratio between the peak current with and without pre-pulse (I_2/I_1) for each condition. Statistical significance was evaluated by ANOVA (versus Control).*

We next evaluated if the basal Ca_V2.2 current inhibition caused by D2R-GHSR co-expression involves GHSR constitutive activity as we observed for the reduction in dopamine evoked Ca_V2.2 currents. We measured basal Ca_V2.2 current levels in cells expressing D2R or both receptors and evaluated the effect of either pre-incubating the cells with the inverse agonist SPA or replacing GHSR with the mutant GHSRA204E [5, 22, 28, 31]. We found that under both of these conditions, the Ca_V2.2 current is restored to control levels (that of D2R-expressing cells) (figure 9). These data indicate that GHSR constitutive activity is indeed required for the reduction of basal Ca_V2.2 current by D2R-GHSR co-expression. Next, we explored the intracellular pathways involved in the effect of D2R-GHSR co-expression on basal Ca_V2.2 current and found that G_q protein and the Gβγ subunit are required. Figure 10 displays the basal current reduction caused by D2R-GHSR co-expression and the occlusion of this inhibitory effect by G_q DN or MAS-GRK2-ct. Moreover, we found that co-expression of AT1R, the GqPCR assayed in figure 5, also failed to modify

the basal $Ca_V2.2$ current when co-expressed with D2R ($I_{CaV2.2}$ +D2R=22.21±5.49 pA/pF, n=11 versus $I_{CaV2.2}$ +D2R+AT1R=24.47±4.19 pA/pF, n=18, P=0.7444, Student's t-test). Our previous study showed that GHSR constitutive activity inhibits $Ca_V2.2$ currents by a chronic mechanism that involves $G_{i/o}$ protein and channel density reduction at the plasma membrane, without requiring G_q and Gβγ. Here we found that D2R-GHSR co-expression reduces basal $Ca_V2.2$ current at low GHSR expression levels by a novel signaling cascade. The basal inhibitory effect that we observed involves the same pathways that occlude the acute dopamine-mediated inhibition of $Ca_V2.2$ currents.

Figure 9. Basal reduction of $Ca_V2.2$ calcium currents by GHSR and D2R co-expression requires GHSR constitutive activity. *Representative traces (top) of $Ca_V2.2$ calcium*



current ($I_{CaV2.2}$) from HEK293T cells co-transfected with $Ca_V2.2$ and D2R (+D2R, n=51), and either GHSR (+GHSR, n=34), GHSR and pre-incubated with SPA (+D2R +GHSR +SPA, n=17), or GHSRA204E (+D2R +A204E, n=60). Bars (bottom) represent the average $I_{CaV2.2}$ levels for each condition. Statistical significance was evaluated by Kruskal-Wallis and Dunn's post-test (versus +D2R).

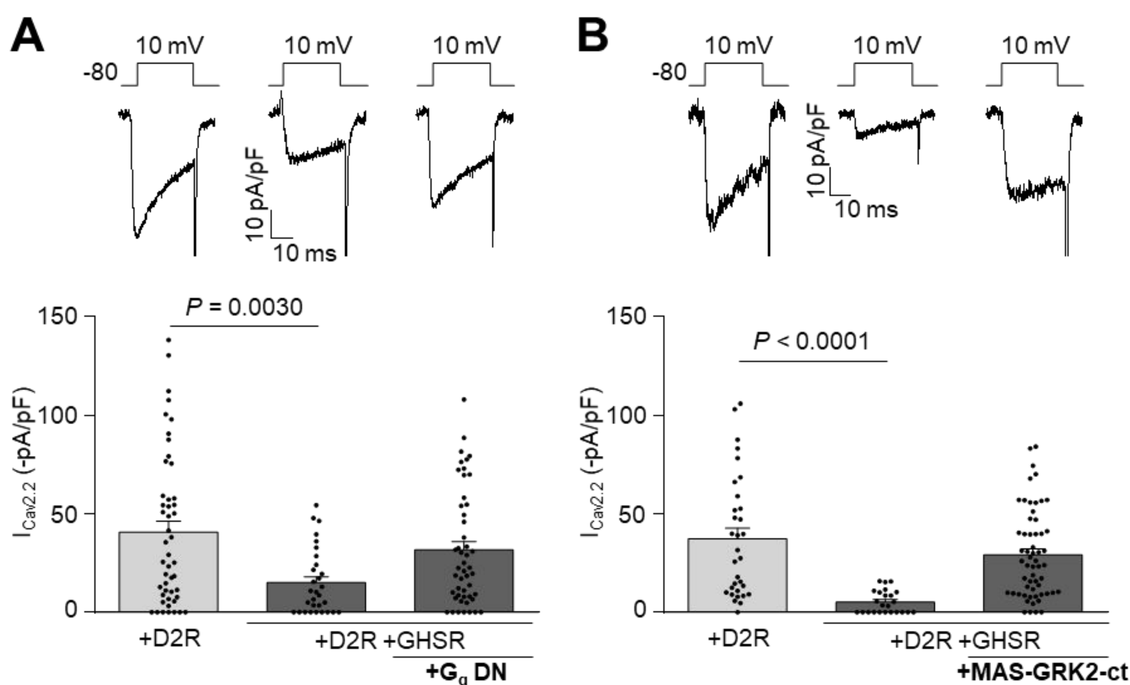


Figure 10. Basal reduction of Ca$_V$2.2 calcium currents by GHSR and D2R co-expression requires G$_q$ protein and Gβγ subunit. A. *Representative traces (top) of Ca$_V$2.2 calcium current ($I_{CaV2.2}$) from HEK293T cells co-transfected with Ca$_V$2.2 and D2R (+D2R, n=48), and either GHSR (+D2R +GHSR, n=30), or GHSR and G$_q$ DN (+D2R +GHSR +G$_q$ DN, n=49). Bars (bottom) represent the average $I_{CaV2.2}$ levels for each condition.* ***B.*** *Representative traces (top) of $I_{CaV2.2}$ from HEK293T cells co-transfected with Ca$_V$2.2 and D2R (+D2R, n=32), and either GHSR (+D2R +GHSR, n=24) or GHSR and MAS-GRK2-ct (+D2R +GHSR +MAS-GRK2-ct, n=58). Bars (bottom) represent the average $I_{CaV2.2}$ values for each condition. Statistical significance was evaluated by Kruskal-Wallis and Dunn's post-test (versus +D2R).*

In summary we found a novel mechanism for controlling Ca$_V$2.2 currents involving the co-expression of two physiologically relevant GPCRs. We propose that the G$_q$ protein, mainly coupled to GHSR, captures Gβγ subunits from the G$_{i/o}$ protein coupled to D2R and consequently reduces the ability of Gβγ to binds Ca$_V$2.2 and may activates a new signaling cascade that basally inhibits Ca$_V$2.2. Thus, the GHSR-D2R interaction would modify the Gβγ-dependent effects of D2R and GHSR on Ca$_V$2.2. Moreover, the constitutive activation of GHSR via G$_q$ and Gβγ subunits are required to reduce basal Ca$_V$2.2 currents when D2R is co-expressed. A similar interaction involving G$_q$ and Gβγ has been described in studies that have also tested for D2R-GHSR heteromers [12, 23]. Both effects of D2R-GHSR co-expression on Ca$_V$2.2 currents require GHSR constitutive activity. This observation diverges from previous reports on the effects of the interaction between these GPCRs [12].

We demonstrated that co-expression of D2R and GHSR has an impact on calcium currents from native and recombinant Ca$_V$2.2. Our results suggest that the effect of dopamine on presynaptic calcium channels would be impaired by the presence of GHSR in

the presynaptic terminal. Since D2R are located at dopaminergic terminals [42], this effect could lead to reduced negative feedback and thus to less regulation of dopamine release. Moreover, since D2R and GHSR are also expressed in other neuronal types, such as GABAergic [5, 43] and cholinergic neurons [18], this mechanism could also modify the effect of dopamine on other neurotransmitter release. We also found that D2R-GHSR co-expression in a heterologous system reduced basal $Ca_V2.2$ currents. If this mechanism occurs in neurons, it is plausible that less $Ca_V2.2$ current would be available at presynaptic terminals in neurons co-expressing D2R and GHSR. This mechanism could be important in a physiological context such as the anorexigenic effect of dopamine observed in hypothalamic neurons in mice, where the co-expression of D2R-GHSR is required for calcium release from intracellular stores, leading to transcriptional activation [12]. The differential modulation of $Ca_V2.2$ by dopamine in these neurons could contribute to this effect by modifying the neuronal communication to appetite control by dopamine.

Our study leads to the question of how this novel control of $Ca_V2.2$ currents could be regulated in neurons. One possibility is that regulation occurs by changing the amount of D2R-GHSR heteromers formed in neurons. This could be modulated by changes in expression levels of both receptors that have been widely described for both GHSR [44] and D2R [45]. On the other hand, modifying GHSR constitutive activity also would impact the mechanism that we described. In this context, the recently reported LEAP2 peptide [46] gains importance since it has been proposed that it could act as a natural inverse agonist.

Methods

Hypothalamic primary neuronal culture

Wild type C57BL/6 mice were housed at IMBICE animal facility in a 12h light-dark cycle and in a climate-controlled room. Mice were bred with *ad libitum* access to food and water. Hypothalamic neurons were removed from mice at embryonic days 15-17 by orienting brains on the dorsal face and removing the hypothalamus with forceps. Tissue was processed as described in [22, 47]. These protocols were approved by the ethics committee of IMBICE in accordance with the Guide for Care and Use of Laboratory Animals of the National Research Council, USA.

Human embryonic kidney (HEK) 293T cell culture

HEK293T cells were used in all heterologous expression studies and for lentiviral production. Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) and 10% of fetal bovine serum was added (FBS; Internegocios). Every time cells achieved 80% of confluence, they were harvested using citric saline 1X (0.135M KCl, 0.015M sodium citrate) and plated in 22 mm diameter plates for electrophysiology assays and 100 mm diameter dishes for lentiviral production.

HEK293T cell transfection

HEK293T cells were transfected for patch clamp experiments using Lipofectamine 2000 (Invitrogen) and Opti-MEM medium (Invitrogen) according to manufacturer protocols with 2.5 µg of final cDNA. We used 0.2 µg of an eGFP-containing plasmid to identify transfected cells. Plated cells were grown for 24 hours before transfection and 24 hours after transfection to achieve appropriate expression levels.

HEK293T cells were transfected for lentiviral production with Polyethylenimine (PEI) (cat 23966, Polysciences, Inc, USA) and Opti-MEM medium using 20 µg of cDNA / 40 µl of PEI. Plated cells were grown for 24 hours before transfection and 48 hours after transfection to allow for lentiviral production.

Plasmids containing the following were used for patch clamp experiments in HEK293T cells: $Ca_V2.2$ (#AF055477), $Ca_V\beta_3$ (#M88751), $Ca_V\alpha_2\delta_1$ (#AF286488), (all Ca_V subunits generously provided by Dr. Diane Lipscombe, Department of Neurosciences, Brown University, Providence, USA), D2R (MG226860, Origene, donated by Dr. Marcelo Rubinstein, INGEBI, Buenos Aires, Argentina), GHSR (GHSR, #AY429112, provided by Dr. J. Marie, Université de Montpellier, Montpellier, France) and AT1R (Donated by Mark Shapiro, University of Texas Health, San Antonio, USA). In indicated experiments we used a 0.1 molar ratio of a point mutant of GHSR (GHSRA204E [5]), a G_q dominant-negative mutant (G_q DN) (G_q-Q209L/D277N; Missouri S&T cDNA Resource Center, Rolla, MO, [36, 48]), or a C-terminal GPCR kinase 2 (MAS-GRK2-ct, gift from S.R. Ikeda, US National Institutes of Health [38, 39]). Ca_V subunits were transfected together in every experiment, each in a 1, 0.1, or 0.5 molar ratio of GPCR:$Ca_V2.2$. We fulfilled the amount of cDNA to 1 molar ratio with the empty plasmid pcDNA3.1(+) (Invitrogen), except in the condition using both GPCRs at 0.5 GPCR:$Ca_V2.2$ molar ratio.

For third generation lentiviral system formation, we used a total of 20 µg of cDNA divided as follows: 3.5 µg of the envelope plasmid pCMV-VSV-G, 4 µg of packaging plasmid pMDLg/pRRE, 2.5 µg of packaging plasmids pRSV-Rev, and 10 µg of transfer vector plasmid FU-GHSR-mOrange-W [5] or FU-D2R-GFP-W.

Transduction

Lentiviral particles were produced as described in [22]. Lentiviral particles containing D2R or GHSR vector were generated and used to infect hypothalamic neurons on day 5 of culture. To over-express both receptors, neurons were infected with both lentiviral particles. Patch clamp experiments were performed nine days after infection.

Drugs

Ghrelin (Global Peptide, cat# PI-G-03) was used as a GHSR agonist. D2R-specific agonists (−) Quinpirole hydrochloride (Q102, Sigma-Aldrich) and Dopamine hydrochloride (H8502, Sigma-Aldrich) were used in neurons and HEK293T cells, respectively. [D-Arg1,D-Phe5,D-Trp7,9,Leu11]–substance P analog (SPA, Santa Cruz Biotechnology, Inc.) was used as a GHSR inverse agonist. Angiotensin II was used in HEK293T cells (A9525, Sigma-Aldrich).

Electrophysiology

Transfected HEK293T cells were detached from plates using trypsin (0.25 mg/mL). DMEM + 10% FBS was then added to inhibit trypsin activity, after which cells were rinsed twice with DMEM. During patch clamp experiments cells were kept at room temperature (23 °C).

Whole-cell voltage clamp configuration mode was applied in all experiments. Depolarizing pulses to 0 mV from a holding potential of -80 mV (for neurons) or to 10 mV from -100 mV (for HEK293T cells) were used to evoke calcium currents for 30 ms every 10 seconds. For pre-pulse protocol we used the same as [24]. Pipette resistance range was 2-5 MΩ, and series resistances lower than 3 times the pipette resistance were admitted.

Currents were recorded using Axopatch 200 amplifier and PCLAMP8.2 (Molecular Devices) or an EPC7 amplifier and PatchMaster (HEKA) sampled at 20 kHz and filtered at 10 kHz (-3dB). Cells with a leak current higher than 100 pA at -100 mV were discarded and leak current was subtracted online using a P/-4 protocol. Perfusion was done by gravity using 10 ml syringes containing bath solution placed 30 cm over the patch clamp chamber. The flow rate was ~1 ml/min. Control, quinpirole, ghrelin, dopamine and angiotensin II containing solutions were applied by different ports connected to the chamber. The liquid junction potential between the internal and the external solution containing 2 mM $CaCl_2$ (see below) was ~7.4 mV and between the internal and the external solution containing 10 mM $BaCl_2$ solution (see below) was ~6.5 mV. These values were measured before experiments and non-corrected thus all voltages showed were slightly more negative. All recordings were obtained at room temperature (~24 °C).

Neurons were kept in high sodium maintenance solution until whole-cell configuration was achieved and sodium currents were observed. Once sodium currents were stabilized, the bath solution was changed to 10 mM barium- and TTX-containing solution to record barium currents. Once barium currents reached a stable baseline, external solution with ghrelin or quinpirole was applied in a random order. For recordings in neurons ground electrode was placed in a separated compartment containing 2 M KCl connected with the recording chamber containing bath solution by a 2 M KCl-agar salt bridge to avoid change on junction potentials due to bath exchange during recordings.

- Internal pipette solution (in mM): 134 CsCl, 10 EGTA, 1 EDTA, 10 HEPES and 4 MgATP (pH 7.2 with CsOH).
- External calcium solution (2 mM) for HEK293T cells (in mM): 140 choline chloride, 10 HEPES, 1 $MgCl_2.6H_2O$ and 2 $CaCl_2.2H_2O$ (pH 7.3-7.4 with CsOH).
- External barium solution (10 mM) for neurons (in mM): 110 choline chloride, 10 HEPES, 10 glucose, 20 tetraethylammonium chloride, 1 $MgCl_2.6H_2O$, 10 $BaCl_2.2H_2O$ and 0.001 tetrodotoxin (TTX; Sigma-Aldrich) (pH 7.3-7.4 with CsOH).
- High sodium maintenance solution (135 mM) for neurons (in mM): 10 HEPES, 1.2 $MgCl_2.6H_2O$, 2.5 $CaCl_2.2H_2O$, 4.7 KCl, 10 glucose and 135 NaCl (pH 7.3-7.4 with NaOH).

Analysis and Statistics

All currents were analyzed using pClampFit 10 software (Molecular Devices) at minimum peak current during test pulse. In experiments studying agonist-evoked activity,

currents were normalized to the maximum value for each condition. In experiments studying basal currents, currents were normalized based on the capacitance of each cell, which represents the size of the cells. Representative traces of currents for each condition were plotted using OriginPro 9 software.

Black dots on figures represent individual data points, and bars with error bars display mean ± SEM.

Statistical analysis was performed using GraphPad Prism 6 software. Kolmogorov–Smirnov, Bartlett's and Brown-Forsythe´s tests were used for population analysis. The statistical tests used for each data set are specified in figure legends. Statistical significance for all tests is 0.05. P-values above 0.05 were omitted. Concentration-response curves were fitted using the Hill equation, and parameter comparison was achieved with GraphPad.

Abbreviations

Voltage-gated calcium channels (Ca_V), G protein-couple receptor (GPCR), dopamine type 2 receptor (D2R), growth hormone secretagogue receptor (GHSR), Förster resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET), adenylate cyclase (AC), phospholipase C (PLC), complementary deoxyribonucleic acid (cDNA), human embryonic kidney (HEK), [D-Arg1,D-Phe5,D-Trp7,9,Leu11]–substance P analog (SPA), G protein-activated inward rectifier potassium channels (GIRK), G_q dominant negative protein (G_q DN), C-terminal GPCR kinase 2 (MAS-GRK-ct), gamma-Aminobutiric acid (GABA), liver-expressed antimicrobial peptide 2(LEAP2), Angiotensin II type 1 receptor (AT1R).

Author Information

SCG, ERM, SSR and JR: Electrophysiology Laboratory of the Multidisciplinary Institute of Cell Biology (IMBICE) [Argentine Research Council (CONICET), Scientific Research Commission of the Providence of Buenos Aires (CIC-PBA) and National University of La Plata (UNLP)], Calle 526 S/N entre 10 y 11, 1900 La Plata, Buenos Aires, Argentina.

MP: Neurophysiology Laboratory of the Multidisciplinary Institute of Cell Biology (IMBICE) [Argentine Research Council (CONICET), Scientific Research Commission of the Providence of Buenos Aires (CIC-PBA) and National University of La Plata (UNLP)], Calle 526 S/N entre 10 y 11, 1900 La Plata, Buenos Aires, Argentina.

Author Contributions

SCG and ERM performed the experiments and data analysis. SSR cloned the FU-D2R-GFP-W plasmid, produced the lentivirus and transduced neuronal cultures. SCG, ERM and JR designed the experiments and wrote the manuscript. MP designed the internalization assay and edit the final version of the manuscript. All authors reviewed the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgment

We thanks Cambria Chou-Freed for carefully reading and editing the manuscript. This work was supported by the grant of the National Agency of Scientific and Technological Promotion of Argentina (PICT 2015-3330, PICT 2017- 0602), National University of La Plata (X765 and X860), and Argentine Research Council (Institutional grant PUE 2017).

Supporting information

The supporting information includes supplementary figure S1 confirming the interaction between D2R and GHSR in our experimental system by flow cytometry and internalization assays and supplementary figure S2 demonstrating that increasing GPCRs cDNA amount in the transfection mix leads to higher protein expression levels in HEK293T cells. These two figures are accompanied by a brief supplementary method section.

References

1. Gerber, K.J., K.E. Squires, and J.R. Hepler, *Roles for Regulator of G Protein Signaling Proteins in Synaptic Signaling and Plasticity.* Mol Pharmacol, 2016. **89**(2): p. 273-86.
2. Leung, C.C.Y. and Y.H. Wong, *Role of G Protein-Coupled Receptors in the Regulation of Structural Plasticity and Cognitive Function.* Molecules, 2017. **22**(7): p. 1239.
3. Ni, Q., S. Mehta, and J. Zhang, *Live-cell imaging of cell signaling using genetically encoded fluorescent reporters.* FEBS J, 2018. **285**(2): p. 203-219.
4. Sato, J., N. Makita, and T. Iiri, *Inverse agonism: the classic concept of GPCRs revisited [Review].* Endocr J, 2016. **63**(6): p. 507-14.
5. Lopez Soto, E.J., F. Agosti, A. Cabral, E.R. Mustafa, V.M. Damonte, M.A. Gandini, S. Rodriguez, D. Castrogiovanni, R. Felix, M. Perello, and J. Raingo, *Constitutive and ghrelin-dependent GHSR1a activation impairs CaV2.1 and CaV2.2 currents in hypothalamic neurons.* J Gen Physiol, 2015. **146**(3): p. 205-19.
6. Meye, F.J., G.M. Ramakers, and R.A. Adan, *The vital role of constitutive GPCR activity in the mesolimbic dopamine system.* Transl Psychiatry, 2014. **4**: p. e361.
7. Sandhu, M., A.M. Touma, M. Dysthe, F. Sadler, S. Sivaramakrishnan, and N. Vaidehi, *Conformational plasticity of the intracellular cavity of GPCR-G-protein complexes leads to G-protein promiscuity and selectivity.* Proc Natl Acad Sci U S A, 2019. **116**(24): p. 11956-11965.
8. Okashah, N., Q. Wan, S. Ghosh, M. Sandhu, A. Inoue, N. Vaidehi, and N.A. Lambert, *Variable G protein determinants of GPCR coupling selectivity.* Proc Natl Acad Sci U S A, 2019. **116**(24): p. 12054-12059.
9. Hermans, E., *Biochemical and pharmacological control of the multiplicity of coupling at G-protein-coupled receptors.* Pharmacol Ther, 2003. **99**(1): p. 25-44.
10. Borroto-Escuela, D.O., J. Carlsson, P. Ambrogini, M. Narvaez, K. Wydra, A.O. Tarakanov, X. Li, C. Millon, L. Ferraro, R. Cuppini, S. Tanganelli, F. Liu, M. Filip, Z. Diaz-Cabiale, and K. Fuxe, *Understanding the Role of GPCR Heteroreceptor Complexes in Modulating the Brain Networks in Health and Disease.* Front Cell Neurosci, 2017. **11**: p. 37.
11. Farran, B., *An update on the physiological and therapeutic relevance of GPCR oligomers.* Pharmacol Res, 2017. **117**: p. 303-327.
12. Kern, A., R. Albarran-Zeckler, H.E. Walsh, and R.G. Smith, *Apo-ghrelin receptor forms heteromers with DRD2 in hypothalamic neurons and is essential for anorexigenic effects of DRD2 agonism.* Neuron, 2012. **73**(2): p. 317-32.

13. Van Craenenbroeck, K., *GPCR oligomerization: contribution to receptor biogenesis.* Subcell Biochem, 2012. **63**: p. 43-65.
14. Fuxe, K., A. Tarakanov, W. Romero Fernandez, L. Ferraro, S. Tanganelli, M. Filip, L.F. Agnati, P. Garriga, Z. Diaz-Cabiale, and D.O. Borroto-Escuela, *Diversity and Bias through Receptor-Receptor Interactions in GPCR Heteroreceptor Complexes. Focus on Examples from Dopamine D2 Receptor Heteromerization.* Front Endocrinol (Lausanne), 2014. **5**: p. 71.
15. Rediger, A., C.L. Piechowski, K. Habegger, A. Gruters, H. Krude, M.H. Tschop, G. Kleinau, and H. Biebermann, *MC4R dimerization in the paraventricular nucleus and GHSR/MC3R heterodimerization in the arcuate nucleus: is there relevance for body weight regulation?* Neuroendocrinology, 2012. **95**(4): p. 277-88.
16. Beaulieu, J.M., S. Espinoza, and R.R. Gainetdinov, *Dopamine receptors - IUPHAR Review 13.* Br J Pharmacol, 2015. **172**(1): p. 1-23.
17. Yan, Z., W.J. Song, and J. Surmeier, *D2 dopamine receptors reduce N-type Ca2+ currents in rat neostriatal cholinergic interneurons through a membrane-delimited, protein-kinase-C-insensitive pathway.* J Neurophysiol, 1997. **77**(2): p. 1003-15.
18. Momiyama, T. and E. Koga, *Dopamine D(2)-like receptors selectively block N-type Ca(2+) channels to reduce GABA release onto rat striatal cholinergic interneurones.* J Physiol, 2001. **533**(Pt 2): p. 479-92.
19. Momiyama, T., *Parallel decrease in omega-conotoxin-sensitive transmission and dopamine-induced inhibition at the striatal synapse of developing rats.* J Physiol, 2003. **546**(Pt 2): p. 483-90.
20. Kisilevsky, A.E. and G.W. Zamponi, *D2 dopamine receptors interact directly with N-type calcium channels and regulate channel surface expression levels.* Channels (Austin), 2008. **2**(4): p. 269-77.
21. Mustafa, E.R., E.J. Lopez Soto, V. Martinez Damonte, S.S. Rodriguez, D. Lipscombe, and J. Raingo, *Constitutive activity of the Ghrelin receptor reduces surface expression of voltage-gated Ca(2+) channels in a CaVbeta-dependent manner.* J Cell Sci, 2017. **130**(22): p. 3907-3917.
22. Martinez Damonte, V., S.S. Rodriguez, and J. Raingo, *Growth hormone secretagogue receptor constitutive activity impairs voltage-gated calcium channel-dependent inhibitory neurotransmission in hippocampal neurons.* J Physiol, 2018. **596**(22): p. 5415-5428.
23. Damian, M., V. Pons, P. Renault, C. M'Kadmi, B. Delort, L. Hartmann, A.I. Kaya, M. Louet, D. Gagne, K. Ben Haj Salah, S. Denoyelle, G. Ferry, J.A. Boutin, R. Wagner, J.A. Fehrentz, J. Martinez, J. Marie, N. Floquet, C. Gales, S. Mary, H.E. Hamm, and J.L. Baneres, *GHSR-D2R heteromerization modulates dopamine signaling through an effect on G protein conformation.* Proc Natl Acad Sci U S A, 2018. **115**(17): p. 4501-4506.
24. Evans, R.M., H. You, S. Hameed, C. Altier, A. Mezghrani, E. Bourinet, and G.W. Zamponi, *Heterodimerization of ORL1 and opioid receptors and its consequences for N-type calcium channel regulation.* J Biol Chem, 2010. **285**(2): p. 1032-40.
25. Schellekens, H., W.E. van Oeffelen, T.G. Dinan, and J.F. Cryan, *Promiscuous dimerization of the growth hormone secretagogue receptor (GHS-R1a) attenuates ghrelin-mediated signaling.* J Biol Chem, 2013. **288**(1): p. 181-91.
26. Wallace Fitzsimons, S.E., B. Chruscicka, C. Druelle, P. Stamou, K. Nally, T.G. Dinan, J.F. Cryan, and H. Schellekens, *A ghrelin receptor and oxytocin receptor heterocomplex impairs oxytocin mediated signalling.* Neuropharmacology, 2019. **152**: p. 90-101.
27. Barrile, F., C. M'Kadmi, P.N. De Francesco, A. Cabral, G. Garcia Romero, E.R. Mustafa, S. Cantel, M. Damian, S. Mary, S. Denoyelle, J.L. Baneres, J. Marie, J. Raingo, J.A. Fehrentz, and

M. Perello, *Development of a novel fluorescent ligand of growth hormone secretagogue receptor based on the N-Terminal Leap2 region.* Mol Cell Endocrinol, 2019. **498**: p. 110573.
28. Pantel, J., M. Legendre, S. Cabrol, L. Hilal, Y. Hajaji, S. Morisset, S. Nivot, M.P. Vie-Luton, D. Grouselle, M. de Kerdanet, A. Kadiri, J. Epelbaum, Y. Le Bouc, and S. Amselem, *Loss of constitutive activity of the growth hormone secretagogue receptor in familial short stature.* J Clin Invest, 2006. **116**(3): p. 760-8.
29. Mary, S., M. Damian, M. Louet, N. Floquet, J.A. Fehrentz, J. Marie, J. Martinez, and J.L. Baneres, *Ligands and signaling proteins govern the conformational landscape explored by a G protein-coupled receptor.* Proc Natl Acad Sci U S A, 2012. **109**(21): p. 8304-9.
30. Damian, M., S. Mary, M. Maingot, C. M'Kadmi, D. Gagne, J.P. Leyris, S. Denoyelle, G. Gaibelet, L. Gavara, M. Garcia de Souza Costa, D. Perahia, E. Trinquet, B. Mouillac, S. Galandrin, C. Gales, J.A. Fehrentz, N. Floquet, J. Martinez, J. Marie, and J.L. Baneres, *Ghrelin receptor conformational dynamics regulate the transition from a preassembled to an active receptor:Gq complex.* Proc Natl Acad Sci U S A, 2015. **112**(5): p. 1601-6.
31. Inoue, H., N. Kangawa, A. Kinouchi, Y. Sakamoto, C. Kimura, R. Horikawa, Y. Shigematsu, M. Itakura, T. Ogata, K. Fujieda, and C. Japan Growth Genome, *Identification and functional analysis of novel human growth hormone secretagogue receptor (GHSR) gene mutations in Japanese subjects with short stature.* J Clin Endocrinol Metab, 2011. **96**(2): p. E373-8.
32. Gingrich, J.A. and M.G. Caron, *Recent advances in the molecular biology of dopamine receptors.* Annu Rev Neurosci, 1993. **16**: p. 299-321.
33. Wacker, D., R.C. Stevens, and B.L. Roth, *How Ligands Illuminate GPCR Molecular Pharmacology.* Cell, 2017. **170**(3): p. 414-427.
34. Kuzhikandathil, E.V., W. Yu, and G.S. Oxford, *Human dopamine D3 and D2L receptors couple to inward rectifier potassium channels in mammalian cell lines.* Mol Cell Neurosci, 1998. **12**(6): p. 390-402.
35. Hernandez-Lopez, S., T. Tkatch, E. Perez-Garci, E. Galarraga, J. Bargas, H. Hamm, and D.J. Surmeier, *D2 dopamine receptors in striatal medium spiny neurons reduce L-type Ca2+ currents and excitability via a novel PLC[beta]1-IP3-calcineurin-signaling cascade.* J Neurosci, 2000. **20**(24): p. 8987-95.
36. Lauckner, J.E., B. Hille, and K. Mackie, *The cannabinoid agonist WIN55,212-2 increases intracellular calcium via CB1 receptor coupling to Gq/11 G proteins.* Proc Natl Acad Sci U S A, 2005. **102**(52): p. 19144-9.
37. Yamada, E., T. Endoh, and T. Suzuki, *Angiotensin II-induced inhibition of calcium currents via G(q/11)-protein involving protein kinase C in hamster submandibular ganglion neurons.* Neurosci Res, 2002. **43**(2): p. 179-89.
38. Raingo, J., A.J. Castiglioni, and D. Lipscombe, *Alternative splicing controls G protein-dependent inhibition of N-type calcium channels in nociceptors.* Nat Neurosci, 2007. **10**(3): p. 285-92.
39. Kammermeier, P.J. and S.R. Ikeda, *Expression of RGS2 alters the coupling of metabotropic glutamate receptor 1a to M-type K+ and N-type Ca2+ channels.* Neuron, 1999. **22**(4): p. 819-29.
40. Ross, E.M., *Signal sorting and amplification through G protein-coupled receptors.* Neuron, 1989. **3**(2): p. 141-52.
41. Zhang, B., A. Albaker, B. Plouffe, C. Lefebvre, and M. Tiberi, *Constitutive activities and inverse agonism in dopamine receptors.* Adv Pharmacol, 2014. **70**: p. 175-214.
42. Beaulieu, J.M. and R.R. Gainetdinov, *The physiology, signaling, and pharmacology of dopamine receptors.* Pharmacol Rev, 2011. **63**(1): p. 182-217.

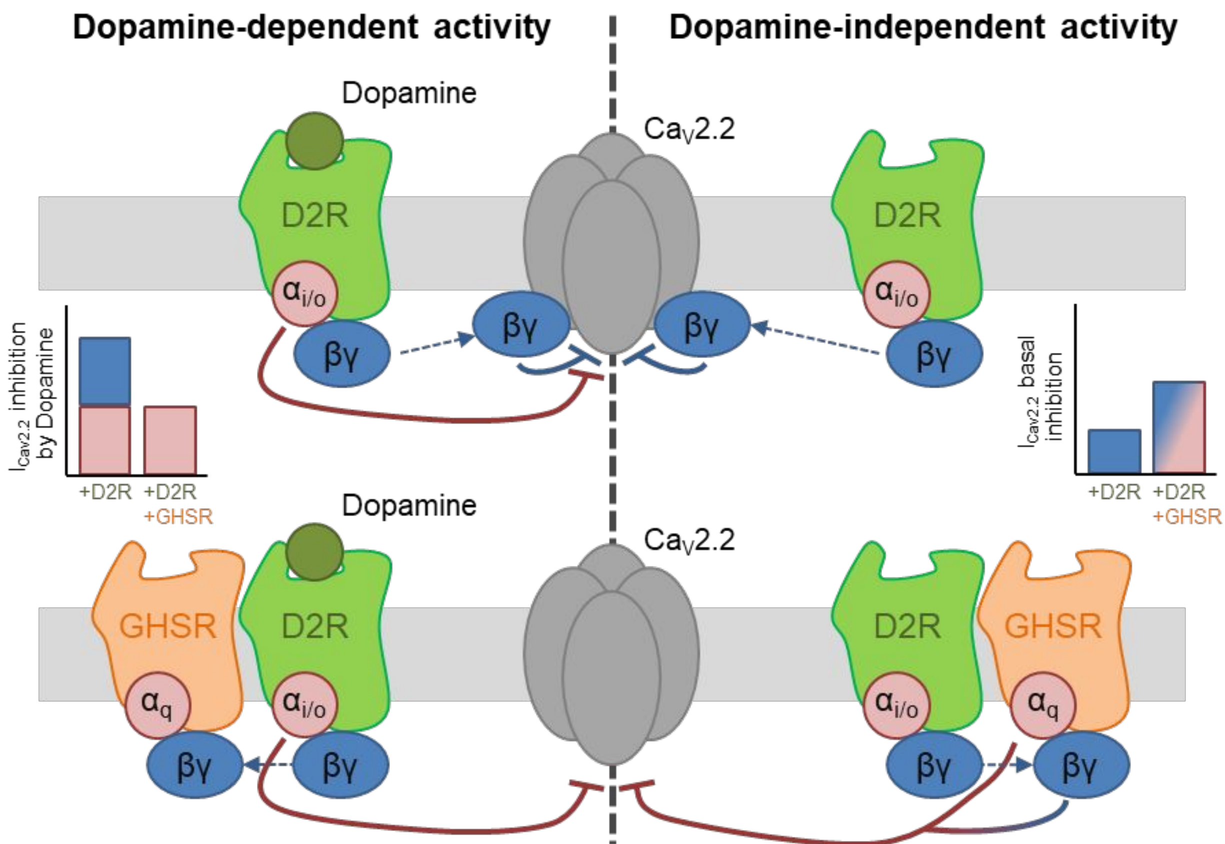
43. Wang, Q., C. Liu, A. Uchida, J.C. Chuang, A. Walker, T. Liu, S. Osborne-Lawrence, B.L. Mason, C. Mosher, E.D. Berglund, J.K. Elmquist, and J.M. Zigman, *Arcuate AgRP neurons mediate orexigenic and glucoregulatory actions of ghrelin.* Mol Metab, 2014. **3**(1): p. 64-72.
44. Fernandez, G., A. Cabral, M.F. Andreoli, A. Labarthe, C. M'Kadmi, J.G. Ramos, J. Marie, J.A. Fehrentz, J. Epelbaum, V. Tolle, and M. Perello, *Evidence Supporting a Role for Constitutive Ghrelin Receptor Signaling in Fasting-Induced Hyperphagia in Male Mice.* Endocrinology, 2018. **159**(2): p. 1021-1034.
45. Johnson, P.M. and P.J. Kenny, *Dopamine D2 receptors in addiction-like reward dysfunction and compulsive eating in obese rats.* Nat Neurosci, 2010. **13**(5): p. 635-41.
46. M'Kadmi, C., A. Cabral, F. Barrile, J. Giribaldi, S. Cantel, M. Damian, S. Mary, S. Denoyelle, S. Dutertre, S. Peraldi-Roux, J. Neasta, C. Oiry, J.L. Baneres, J. Marie, M. Perello, and J.A. Fehrentz, *N-Terminal Liver-Expressed Antimicrobial Peptide 2 (LEAP2) Region Exhibits Inverse Agonist Activity toward the Ghrelin Receptor.* J Med Chem, 2019. **62**(2): p. 965-973.
47. Agosti, F., S. Cordisco Gonzalez, V. Martinez Damonte, M.J. Tolosa, N. Di Siervi, H.B. Schioth, C. Davio, M. Perello, and J. Raingo, *Melanocortin 4 receptor constitutive activity inhibits L-type voltage-gated calcium channels in neurons.* Neuroscience, 2017. **346**: p. 102-112.
48. Yu, B. and M.I. Simon, *Interaction of the xanthine nucleotide binding Goalpha mutant with G protein-coupled receptors.* J Biol Chem, 1998. **273**(46): p. 30183-8.

For table of content use only

Dopamine receptor type 2 (D2R) and ghrelin receptor (GHSR) co-expression alters $Ca_V2.2$ modulation by G protein signaling cascades.

Santiago Cordisco Gonzalez[1], Emilio R. Mustafá[1], Silvia S. Rodriguez[1], Mario Perello[2] and Jesica Raingo[1*]

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29



1

30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60