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MELANOCORTIN 4 RECEPTOR CONSTITUTIVE ACTIVITY INHIBITS L-TYPE VOLTAGE-GATED CALCIUM CHANNELS IN NEURONS

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Abstract—The melanocortin 4 receptor (MC4R) is a G protein-coupled receptor (GPCR) that is expressed in several brain nuclei playing a crucial role in the regulation of energy balance controlling the homeostasis of the organism. It displays both agonist-evoked and constitutive activity, and moreover, it can couple to different G proteins. Most of the research on MC4R has been focused on agonist-induced activity, while the molecular and cellular basis of MC4R constitutive activity remains scarcely studied. We have previously shown that neuronal N-type voltage-gated calcium channels ($Ca_V2.2$) are inhibited by MC4R agonist-dependent activation, while the Ca_V subtypes that carry L- and P/Q-type current are not. Here, we tested the hypothesis that MC4R constitutive activity can affect Ca_V, with focus on the channel subtypes that can control transcriptional activity coupled to depolarization (L-type, $Ca_V1.2/1.3$) and neurotransmitter release (N- and P/Q-type, $Ca_V2.2$ and $Ca_V2.1$). We found that MC4R constitutive activity inhibits specifically $Ca_V1.2/1.3$ and $Ca_V2.1$ subtypes of Ca_V. We also explored the signaling pathways mediating this inhibition, and thus propose that agonist-dependent and basal MC4R activation modes signal differentially through G_s and $G_{i/o}$ pathways to impact on different Ca_V subtypes. In addition, we found that chronic incubation with MC4R endogenous inverse agonist, agouti and agouti-related peptide (AgRP), occludes Ca_V inhibition in a cell line and in amygdaloid complex cultured neurons as well. Thus, we define new mechanisms of control of the main mediators of depolarization-induced calcium entry into neurons by a GPCR that displays constitutive activity.

Key words: calcium channels, melanocortin receptor, electrophysiology, G protein, amygdala.

INTRODUCTION

The melanocortin 4 receptor (MC4R) plays crucial roles in regulation of energy balance, and mutations in the MC4R gene are the most common cause of monogenic obesity, accounting for 2–5% of all cases (Hinney et al., 1999; Vaisse et al., 2000). A large body of evidence shows that genetic or pharmacological manipulations of the neuronal MC4R signaling in rodents strongly affect satiety and/or energy expenditure regulation impacting on body weight (Kask et al., 1998). The indubitable role of MC4R central signaling in the regulation of body weight has supported its study as a potential therapeutic target for weight loss in humans.

MC4R is a G protein-coupled receptor (GPCR) that is activated by the melanocortin peptides including alpha- and beta-melanocyte stimulating hormones (α-MSH and ß-MSH, respectively). An interesting feature of MC4R is its strong constitutive activity that makes it capable to signal in a ligand-independent manner. In addition, the melanocortin receptors are unique since they are the only known GPCRs whose activity can be reduced by endogenous peptides, i.e. agouti and agouti-related peptide (AgRP). Although agouti and AgRP were initially described to function as selective antagonists that prevent binding of α-MSH, it was later recognized that AgRP also exhibits inverse agonist properties inhibiting the constitutive activity of MC4R in the absence of α-MSH (Haskell-Luevano and Monck, 2001; Nijenhuis et al., 2001). AgRP-producing neurons are exclusively located at the hypothalamic arcuate nucleus and are known to be implicated in several physiological functions (Padilla et al., 2016). One of the key targets of the AgRP-producing neurons is the amygdala that, together with the hypothalamic paraventricular nucleus (PVN), is the brain area with the highest MC4R expression levels (Gantz et al., 1993; Kishi et al., 2003; Liu et al., 2003).

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Abbreviations: AgRP, agouti-related peptide; α-MSH, alpha-melanocyte stimulating hormone; ß-MSH, beta-melanocyte stimulating hormone; Ca_V, calcium channels; cAMP, cyclic AMP; ChTx, cholera toxin; eGFP, enhanced green fluorescent protein; GPCR, G protein-coupled receptor; MC4R, melanocortin 4 receptor; PVN, paraventricular nucleus; PTx, pertussis toxin; PKA, protein kinase A.

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Importantly, MC4R signaling exclusively in the PVN and amygdala is sufficient to restore food intake and prevent the obesity in a MC4R-deficient mouse model (Balthasar et al., 2005).

The classical signaling pathway for the agonist-induced MC4R activation involves coupling to the heterotrimeric stimulatory G protein (G_s), further increases of cyclic AMP (cAMP) production, and the consequent activation of the protein kinase A (PKA) (Gantz et al., 1993; Gao et al., 2003; Shinyama et al., 2003). Utilizing this pathway, MC4R activation is capable of controlling internal calcium concentration in HEK293 cells expressing this receptor by inducing calcium release from internal compartments (Mountjoy et al., 2001) as well as by inhibiting N-type voltage-gated calcium currents (Agosti et al., 2014). On the other hand, α-MSH-induced MC4R activation stimulates the pertussis toxin (PTx)-sensitive GTPγS binding in a hypothalamic cell line (GT1-7 cells) suggesting that MC4R is also able to couple to $G_{i/o}$ proteins (Buch et al., 2009). MC4R has been shown to also activate G protein-independent pathways, including the MAPK and ERK1/2 pathway (Vongs et al., 2004; Berthoud et al., 2006; Chai et al., 2006; Mo and Tao, 2013). On the other hand, a recent report demonstrated that MC4R can control neuronal membrane potential and firing in a G protein-independent manner. This regulation is due to the opposite effect of MC4R agonist (inhibition) and inverse agonist (activation) on inward rectifying potassium currents in hypothalamic neurons (Ghamari-Langroudi et al., 2015). Despite this relevant work, our understanding on the molecular and cellular basis by which the MC4R constitutive activity affects neuronal function is currently unclear.

Here, we tested the hypothesis that MC4R constitutive activity can affect neuronal voltage-gated calcium channels (Ca_V) that are the main mediators of depolarization-induced calcium entry into neurons. Changes in membrane Ca_V density or in its capability to conduct calcium can greatly affect calcium-dependent neuronal functions. In particular, we focused on the Ca_V subtypes that are able to control transcriptional activity coupled to depolarization and neurotransmitter release. We specifically studied $Ca_V1.2$ and $Ca_V1.3$ channels (a.k.a. L-type channels) that are responsible for the calcium influx that couples membrane voltage changes to gene transcription. As expected, these Ca_V subtypes are generally located at dendrites and neuronal soma (Ahlijanian et al., 1990; Westenbroek et al., 1990; Calin-Jageman and Lee, 2008). We also inquired if $Ca_V2.1$ and $Ca_V2.2$, that carry N- and P/Q-type currents in neurons, are modulated by MC4R constitutive activity. These Ca_V subtypes are located at pre-synaptic spots and they are activated during periods of action potential-driven depolarization at the synaptic terminals (Evans and Zamponi, 2006).

GPCR activity is one of the most effective mechanisms to control Ca_V (Catterall, 2000; Currie, 2010). Numerous examples in the literature show the physiological impact and the pathways involved in Ca_V modulation by agonist-mediated activation of GPCRs (Altier and Zamponi, 2008). However, reports on GPCR constitutive activity actions on Ca_V are scarce. In this regard, we have recently demonstrated that the ghrelin receptor (or GHSR1a), another GPCR that also displays a high constitutive activity (Holst and Schwartz, 2004), chronically inhibits Ca_V by reducing its density at the plasma membrane (Lopez Soto et al., 2015). Moreover, our current work about MC4R constitutive activity is a follow up study of another previous report from our lab showing that pre-synaptic $Ca_V2.2$ calcium channels are inhibited by the agonist-dependent activation of MC4R, while the Ca_V subtypes that carry L- and P/Q-type current are not. Now, we extend our study looking at the effect of MC4R constitutive activity on those Ca_V subtypes.

EXPERIMENTAL PROCEDURES

Clones and transient transfections

The MC4R and MC3R cDNAs (taken from the commercial plasmid: Open Biosystems, cat# MMM1013-99829006 and MMM1013-99827302 Huntsville, Alabama, USA) was inserted in the L307 vector by Dr. Mikhail Khvotchev. L307 contains an internal ribosome entry site (Ires) that allows the expression of the enhanced green fluorescent protein (eGFP), which was used to identify transfected cells. The principal subunits of calcium channels ($Ca_V2.2$ (# AF055477), $Ca_V1.2$ (# AY728090), $Ca_V1.3$ (# AF370009), $Ca_V2.1$ (# AY714490)) and the auxiliary subunits $Ca_V\beta_3$ (# M88751) and $Ca_V\alpha_2\delta_1$ (# AF286488) were kindly provided by Dr. Diane Lipscombe (Brown University). The punctual mutation of the MC4R (MC4R-R18C) was performed in the laboratory of Dr. Diane Lipscombe by Dr. Summer Allen.

HEK293 cells were plated and growth with Dulbecco's Modified Eagle Medium (DMEM, cat# P3030, Microvet, Buenos Aires, Argentina) with 10% fetal bovine serum (FBS, cat#1650-01, Internegocios, Mercedes, Buenos Aires, Argentina). 24 h later HEK293 cells were transiently transfected with different MC4/Ca_V molar ratios ranging from 1:1 to 0:1 using Lipofectamine 2000 (cat# 11668019, ThermoFisher Scientific, Waltham, MA, USA). In all experiments the total quantity of DNA transfected was 3 μg. For electrophysiology experiments transfected cells were identified by the expression of eGFP expressed from the MC4R-Ires-eGFP and/or MC3R-Ires-eGFP plasmids. MC3R is a melanocortin receptor with 80% homology with MC4R that lacks constitutive activity. Moreover, when using different MC4R/Ca_V molar ratio, all Ca_V subunits cDNA were constant and MC4R decreased, and MC3R was added proportionally to maintain the total 3 μg. For imaging experiments HEK293 cells were co-transfected with MC4R and $Ca_V1.3$ (with the calcium channel auxiliary subunits $Ca_V\alpha_2\delta_1$ and $Ca_V\beta_3$) in a 0.1, 0.5 and 1 molar ratio, respectively. For imaging experiments all Ca_V subunits cDNA were constant, MC4R decreased, and empty pcDNA vector was added proportionally to maintain the total 3 μg.

Drugs

The Gs inhibitor, cholera toxin (ChTx, cat# C8052, Sigma Aldrich, St. Louis, MO, USA); the $G_{i/o}$ inhibitor, pertussis toxin (PTx, Sigma Aldrich, cat# P7208); the MC4R synthetic agonist, MTII (cat# 043-23, Phoenix Pharmaceutical, Karlsruhe, Baden-Wurtemberg, Germany); the P/Q-type channel blocker, ω-agatoxin-IVA (cat# 4256-s, Peptides International, Ky, USA); the N-type channel blocker, ω-conotoxin-GVIA (cat# C-300, Alomone lab, Jerusalem, Israel); the L-type channel antagonist, nifedipine (cat# N7634 Sigma Aldrich); and the MC4R inverse agonist, Agouti-related peptide (AgRP, cat# 003-57, Phoenix Pharmaceuticals) were used.

Animals

A number of six pregnant C57BL6/J mice were utilized to obtain the neuronal cultures. Animals were bred at the animal facility of the Multidisciplinary Institute of Cell Biology (IMBICE). They were housed in a 12-h light/dark cycle with food and water *ad libitum*. This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All experimentation received approval from the Institutional Animal Care and Use Committee of the IMBICE.

Primary amygdaloid neuronal cultures

Neuronal cultures were obtained from mice on embryonic day 16–17 of both sexes. The procedure protocol was identical to that previously described (Agosti et al., 2014). Briefly, pregnant mice were anesthetized with chloral hydrate (500 mg/kg) to remove the embryos. The embryo brains were exposed and blocks of tissue containing mainly the amygdaloid complex and neighboring piriform cortex from each hemisphere (Swanson and Petrovich, 1998) were placed in Hank's solution, and cells were dissociated with trypsin 0.25 mg/ml (cat#L2700-100, Microvet) and deoxyribonuclease I from bovine pancreas 0.28 mg/ml (cat#D5025, Sigma Aldrich) at 37 °C for 20 min, then 300 μl of FBS was added to stop the digestion and cells were mechanically dissociated. 50,000 cells were plated on 12-mm-diameter glasses previously treated with poly-L-lysine (cat# P8920, Sigma Aldrich) and laid over 15-mm-diameter wells. Cells were incubated at 37 °C in a 95% O_2 and 5% CO_2 atmosphere with DMEM/F12 1:1 medium supplemented with 10% FBS, 0.25% glucose, 2 mM glutamine (cat#21051-016, GIBCO, United States), 3.3 μg/ml insulin (Nordisk Pharm Ind, Inc, Clayton, North Carolina, United States), 5 U/ml penicillin G sodium salt (Richet, Buenos Aires, Argentina), 40 μg/ml gentamicin sulfate salt (Richet), 1% vitamin solution (cat#L2112-100, Microvet) and B27 supplement (1:50) (cat#17504-044, GIBCO) (DMEM-S). On the fourth day in culture half of the incubation medium was replaced with medium containing cytosine β-D-arabinofuranoside (AraC) to reach a final concentration of 5 μM (cat# C1768, Sigma Aldrich). From the sixth day in culture, neurons were use in patch clamp experiments or for immunohistochemistry. When the experiment required, AgRP was added to the culture medium to a final concentration of 100 nM for 44 h.

Electrophysiology

Patch clamp experiments were performed using an Axopatch 200 amplifier (Molecular Devices) at a sample rate of 20 kHz and using a 10 kHz (3 dB) filter. Data were digitalized with a Digidata 1322A (Molecular Devices) and registered using PCLAMP 8.2 software. Electrodes resistances others than 2–4 MΩ were omitted, as well as series resistances more than 6 MΩ. A P/-4 protocol was used for current leak subtraction.

Calcium currents in transiently transfected HEK293 cells. Whole-cell patch-clamp experiments were performed on GFP positive transiently transfected HEK293 cells dispersed with 0.25 mg/ml trypsin, rinsed twice and kept at room temperature in culture media during the patch clamp experimental day. The internal pipette solution contained (in mM): 134 CsCl, 10 EGTA, 1 EDTA, 10 HEPES, 4 MgATP (pH 7.2 with CsOH). The external solution contained (in mM): 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 140 choline chloride (pH 7.4 with CsOH). The test-pulse protocol consisted in square pulses that were applied from −100 mV to 10 mV for 15 ms every 10 s, except for the test-pulse protocol used on $Ca_V1.3$, which was applied to −10 mV instead of +10 mV. Current-voltage relationship (IV) protocol: 5 mV increasing square test pulses of 15 ms of duration were applied ranging from −60 to +80 mV (Raingo et al., 2007).

In order to avoid expression level changes artifacts, we recorded currents alternating among different conditions (blind) in a random order along the experimental day.

Barium currents in primary neuronal cultures. After 6 days in culture neurons were patched in whole-cell mode using the same internal solution as described for HEK293 cells and a high-sodium external solution (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). Once neurons were clamped and held at −80 mV, Ca_V currents were registered. For this, the external solution was replaced by a high-barium solution (in mM): 1 $MgCl_2$, 10 HEPES, 10 glucose, 10 $BaCl_2$, 20 tetraethylammonium chloride, 110 choline chloride and 0.001 tetrodotoxin (cat# T8024, Sigma Aldrich) (pH 7.4 with CsOH). Agar/KCl bridge was used to avoid junction potential due to solution exchange. Test pulses were applied from −80 mV to 0 mV for 20 ms every 10 s. We checked for absence of current run down in the time frame of the experiment presented in Fig. 1. We applied drugs in an identical total time in both experimental conditions (control = 928.00 ± 168.48 and AgRP preincubation = 855.71 ± 52.05 seconds, n.s. *t*-Test).

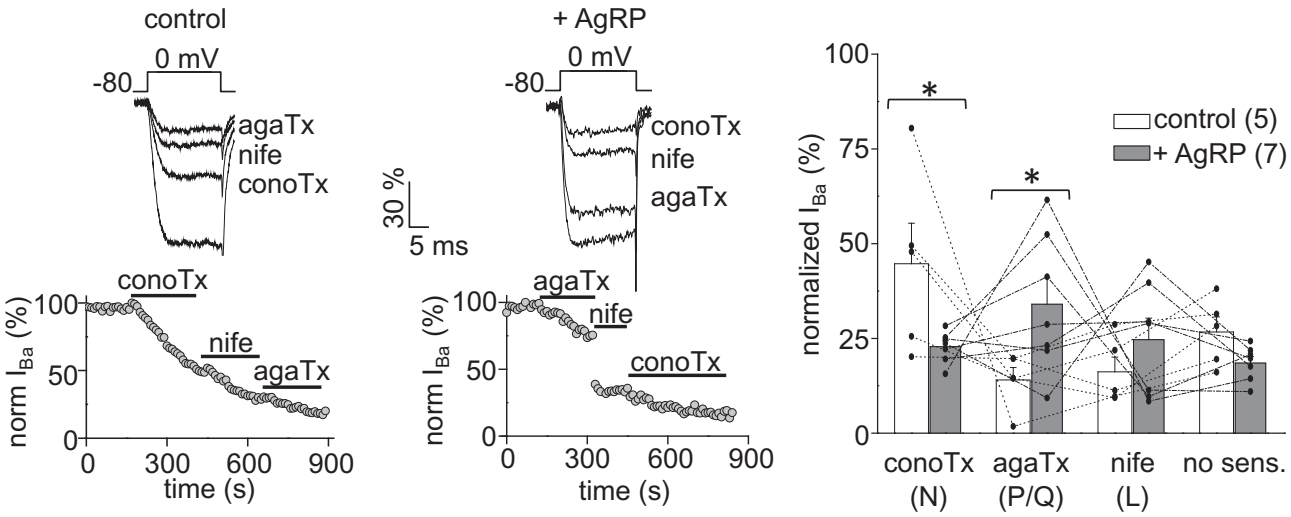

Fig. 1. Effect of reduction of MC4R constitutive activity on voltage-gated calcium currents in cultured amygdaloid neurons. AgRP modifies the profile of native Ca_V currents. Representative traces of normalized barium currents and time courses recorded in amygdaloid neurons in culture in absence or random sequential application of ω-conotoxin-GVIA, ω-agatoxin-IVA and nifedipine (left panel), and the individual effect of toxins on each cell and the average values of the inhibition percentage by each blocker and the no sensitive component of the total current in control or pre-incubated with 100 nM AgRP for 44 h (right panel) (*indicates statistical significant differences among groups by a Student *t*-test, $p < 0.05$).

Imaging

48 h after transfection, culture medium was added with 1 μg/ml of membrane marker (cat# C10045, ThermoFisher Scientific) for 1 min at 37 °C and immediately cells were washed three times with PBS. Fluorescence photomicrographs were obtained with an inverted optical fluorescence microscope (Eclipse Ti; Nikon), equipped with B2A and G2A filters and with a camera (DS-Ri2; Nikon). Analysis of photomicrographs was performed with FIJI free software, using the red fluorescent signal to mark out the plasma membrane and quantify green fluorescence intensity in the total area of each green cell as integrated density. Total fluorescence intensity for each cell was calculated corresponding to the total area.

Immunocytochemistry assay against c-Fos on primary neuronal cultures

Amygdaloid cultured neurons were treated or not with the MC4R inverse agonist, AgRP (100 nM), for 44 h. Then neurons were rinsed 3 times with PBS for 5 min and fixed with formaldehyde 4% for 30 min. After that, they were rinsed once again 3 times with PBS for 5 min and were incubated with the blocking solution (donkey serum 3%/PBS-triton 0.25%) and permeabilized for 1 h at room temperature. Later, they were incubated overnight with the primary antibody anti-c-Fos (1:200, cat# sc-7202, Santa Cruz, TX, USA). Then, they were rinsed 3 times with PBS for 5 min and they were next incubated with the secondary antibody Alexa 488 (1:1000, cat# ab150077, Invitrogen) for 2 h. After rinsing 3 more times with PBS for 5 min, neurons were mounted with Hoechst-mounting solution. Fluorescence photomicrographs were obtained with an inverted optical fluorescence microscope (Eclipse Ti, Nikon), equipped with B2A and G2A filters and with a camera (DS-Ri2; Nikon). Analysis of the images was performed counting the percentage of c-Fos positive (green) neurons over the total number of neurons, identifying neurons by the size and shape of its nucleus.

cAMP assay

cAMP levels measured in transfected HEK293 cells transfected or not (nt) with empty plasmid (+ pcDNA), MC4R (+ MC4R) or mutant MC4R (+ R18C) and exposed to AgRP 100 nM for 44 h. Then, cells were incubated in presence of 10 μM IBMX for 10 min at 37 °C. After this, 25 μM forskolin was added. Following 10-min treatment, supernatants were removed and 0.8 ml of ethanol was added to each well. Ethanol was dried out, and residues suspended in 50 mM Tris-HCl, pH 7.4, 0.1% BSA for further cAMP determination. Cyclic AMP content was determined by a competitive radio-binding assay for PKA using [3H]-cAMP as previously described (Davio et al., 1995). The standard curve was performed using eight cAMP concentrations ranging from 0.1 to 90 pmol. Duplicate samples in at least three independent experiments were analyzed.

Statistics

Data are expressed as mean ± SE, and n values are indicated in brackets in each figure. Normal distribution was tested with D'Agostino–Pearson or Shapiro–Wilk tests, and homogeneity of variances was assessed with Bartlett's or Brown-Forsythe test for normal and non-normal data (GRAPHPAD PRISM 6 software). Statistical significance was assessed with one- or two-sample Student's *t*-tests (OriginPro8, for normal distributed and homogeneous variance sets of data), or with non-parametric tests, i.e. Mann–Whitney test or

Kruskal–Wallis one-way ANOVA (GRAPHPAD PRISM 6 software, for non-normal distributed and non-homogeneous variance datasets), as indicated in each figure. Significant differences were considered when $p < 0.05$.

RESULTS

MC4R constitutive activity modulates the contribution of each Ca_V subtype to the total calcium current in cultured amygdaloid complex neurons

We have previously used mice cultured neurons from the amygdaloid complex to study the role of agonist-induced MC4R signaling on native Ca_V (Agosti et al., 2014) and we have proved that these neurons express high levels of MC4R. Now, we tested if MC4R constitutive activity can affect the total Ca_V current as well as each subtype's overall contribution. For this purpose, we recorded total calcium currents in amygdaloid neurons chronically exposed (44 h), or not, to 100 nM AgRP. On these conditions, we tested the effect of specific Ca_V subtypes blockers applied in a random sequence waiting until the effect was stable in each application. First, we found that neurons chronically exposed to AgRP displayed a larger total calcium current in comparison to control conditions, without reaching statistical significance (control = −64.74 ± 9.59 pA/pF versus AgRP pre-incubation = −84.83 ± 13.96 pA/pF, $n = 5$ and 7 neurons, $p = 0.3038$ *t*-Test). To estimate the contribution of the $Ca_V1.2$/$Ca_V1.3$ (L-type current), $Ca_V2.1$ (P/Q-type current) and $Ca_V2.2$ (N-type current) channels to total current in each condition we used either 1 μM nifedipine (nife), 0.1 μM ω-agatoxin-IVA (agaTx) or 1 μM ω-conotoxin-GVIA (conoTx), respectively. In control condition (in absence of AgRP) we found that conoTx-sensitive currents represent the major contributor to the total calcium currents (44.71 ± 10.67% $n = 5$) and that agaTx- and nifedipine-sensitive currents are minor contributors to the total calcium currents. Interestingly, we found a differential effect of the AgRP treatment on the different Ca_V subtypes since conoTx-sensitive currents were reduced to the 22.80 ± 1.57% ($n = 7$) of the total calcium currents ($p = 0.0358$ vs. control conoTx-sensitive current, Student *t*-test) while agaTx-sensitive increased and nifedipine-sensitive currents tended to increase (Fig. 1, agaTx 14.04 ± 3.28% $n = 5$ vs agaTx AgRP 34.01 ± 6.99% $n = 7$ $p = 0.0470$ Student *t*-test; nife 16.18 ± 3.89% $n = 5$ vs nife AgRP 24.68 ± 5.67% $n = 7$ $p = 0.2858$). Therefore, the presence of AgRP modifies the contribution of different Ca_V subtypes to the total calcium current.

MC4R constitutive activity selectively inhibits $Ca_V1.2$, $Ca_V1.3$ and $Ca_V2.1$ subtypes in HEK293 cells

In order to explore the impact of MC4R constitutive activity in a controlled manner, we used a heterologous expression system in which the MC4R expression level is adjusted by the amount of cDNA in the transfection mix in a dose–response fashion (Lopez Soto et al., 2015). To discard variations in Ca_V expression levels (by changes in transcription, translation or protein trafficking) when different amounts of MC4R are utilized, we co-transfected the Melanocortin 3 Receptor, in a MC3R-Ires-GFP L307 plasmid. Thus we fixed the total amount of plasmid with the MC3R, which shares 76% homology with MC4R and lacks constitutive activity (Kim et al., 2002). Under these conditions, the MC4R gene expression was shown to dose-responsively increase with the amount of plasmid used for transfections, as indicated by the fluorescence signal from the soluble GFP protein expressed in a theoretical 1 to 1 proportion with MC4R by the MC4R-Ires-GFP plasmid (Fig. 2A). In order to examine if the amount of MC4R differentially affects the different Ca_V subtypes, we recorded Ca_V currents from HEK293 cells co-expressing each Ca_V subtype and different amounts of MC4R cDNA. We found that the presence of the MC4R significantly and dose-responsively inhibits $Ca_V1.2$, $Ca_V1.3$ and $Ca_V2.1$ currents (Fig. 2B); being the $Ca_V1.3$ the calcium channel more sensitive to the MC4R co-expression, since the current reduction is already significant at 0.5 MC4R/$Ca_V1.3$ molar ratio. In contrast, we found that the presence of the MC4R failed to affect $Ca_V2.2$ currents in any tested molar ratio. Moreover, we calculated the half activation voltage ($V_{1/2}$) for $Ca_V1.3$ and $Ca_V2.1$ expressing cells with or without MC4R co-expression at 1:1 molar ratio and found not differences ($Ca_V1.3$ $V_{1/2} = -29.93 \pm 0.96$ mV and $Ca_V1.3$ + MC4R $V_{1/2} = -30.13 \pm 3.13$ mV, $p = 0.9475$ and for $Ca_V2.1$ $V_{1/2} = -9.08 \pm 1.31$ mV and $Ca_V2.1$ + MC4R $V_{1/2} = -9.02 \pm 2.65$ mV, $p = 0.9845$, *t*-Test). Thus, MC4R co-expression selectively impacts on some Ca_V subtypes without shifting in its voltage dependency.

MC4R constitutive activity regulates neuronal activity marker c-Fos in amygdaloid neuronal cultures

Since MC4R signaling seemed to regulate Ca_V subtypes involved in the control of the transcription, we tested if the MC4R constitutive signaling impacts on the overall transcriptional activity of the amygdaloid neurons using the neuronal activation marker (c-Fos) expression as readout. We found that cultures chronically exposed to AgRP display a larger number of neurons positive for c-Fos (Fig. 3), consistent with our observation of a reduction of L-type Ca_V function induced by MC4R constitutive activity. This result suggests that MC4R constitutive activity modification of the overall contribution of the different Ca_V subtypes to the total calcium current could impact on the transcriptional activity in amygdaloid neurons.

MC4R constitutive activity inhibits $Ca_V1.3$ currents in a $G_{i/o}$-dependent pathway in HEK293 cells

Since $Ca_V1.3$ seems to be the more sensitive Ca_V targeted by MC4R constitutive activity, we next decided to get insight into the intracellular pathway mediating such inhibitory effect. First, we tested if AgRP is able to occlude the effect of MC4R co-expression on $Ca_V1.3$ current level. For this purpose, we recorded calcium currents in HEK293 cells co-expressing MC4R and functional $Ca_V1.3$ channels, in a 1 MC4R/$Ca_V1.3$ molar ratio, that were chronically (44 h) or acutely exposed to

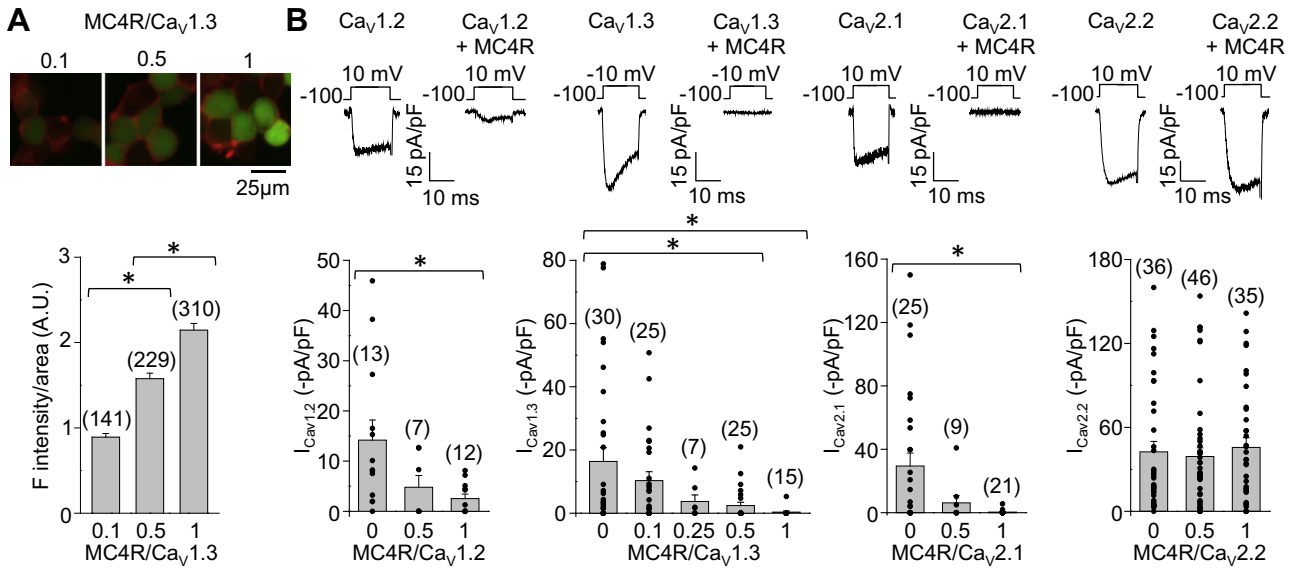

Fig. 2. MC4R co-expression inhibits specific subtypes of Ca_V. (A) GFP signal intensity increases in cells transfected with growing amounts of MC4R-Ires-GFP plasmid. Representative photomicrographs of HEK293 cells transfected with MC4R-Ires-GFP and $Ca_V1.3$ containing plasmids and treated with the red membrane marker Cell-mask, and average green fluorescent intensity per area unit at different MC4R/$Ca_V1.3$ molar ratios (n values are from 3 independent experiments, *indicates statistical significant differences among groups by a Kruskal–Wallis test and Dunn's multiple comparisons test, $p < 0.001$). (B) Specific Ca_V subtypes display a current reduction in presence of increasing amounts of MC4R. Representative traces and average values of -from left to right- $Ca_V1.2$, $Ca_V1.3$, $Ca_V2.1$ and $Ca_V2.2$ currents in presence of increasing MC4R/Ca_V molar ratios (*indicates statistical significant differences among groups by Kruskal–Wallis test and Dunn's multiple comparisons post-test, $p < 0.05$).

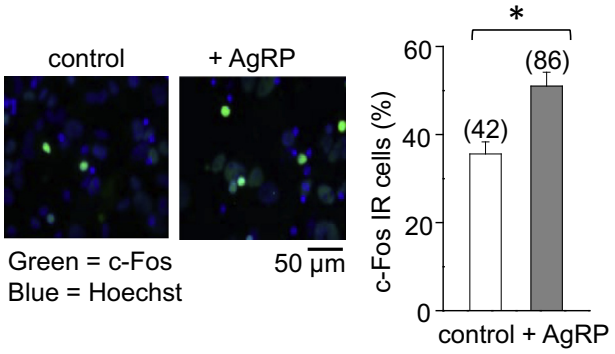

Fig. 3. AgRP increases c-Fos expression. Representative photomicrographs of c-Fos immunocytochemistry on cultured amygdaloid neurons (blue: Hoechst stained nuclei; and green: c-Fos signal) and average percentage of c-Fos immunoreactive cells with or without pre-incubation with 100 nM AgRP (*n* values are the number of fields analyzed; *indicates statistical significant differences among groups by Mann–Whitney *t*-test, $p < 0.05$).

100 nM AgRP. We corroborated that AgRP does not modified the $Ca_V1.3$ currents itself, by assaying the effect of AgRP pre-incubation in cells without MC4R expression ($Ca_V1.3$ = −29.97 ± 4.65 pA/pF, $Ca_V1.3$/AgRP = −23.30 ± 5.07 pA/pF, *t*-Test, n.s.). Fig. 4A shows data verifying the inhibition of basal $Ca_V1.3$ currents by MC4R expression (Dunn's post-test, $P < 0.05$, as compared to Cav1.3 group) and that the chronic AgRP exposure abrogates this effect of MC4R expression ($P > 0.05$ vs $Ca_V1.3$ group). Of note, $Ca_V1.3$ currents in cells co-expressing MC4R did not statistically differed in the presence or the absence of AgRP. On the other hand, the acute application of this inverse agonist failed to modify the $Ca_V1.3$ current reduction by MC4R reduction at all (Fig. 4B). Thus, basal MC4R-induced $Ca_V1.3$ current reduction involves its constitutive activity and requires a long-term mechanism.

In order to investigate the G protein subtype mediating the effect of the MC4R constitutive activity on $Ca_V1.3$ currents, we tested MC4R-induced inhibition in the presence of either the G_s blocker cholera toxin (ChTx, 500 ng/ml) or the $G_{i/o}$ blocker pertussis toxin (PTx, 500 ng/ml). We found that PTx has a tendency to abolish the MC4R-induced inhibition of $Ca_V1.3$ currents (without reaching statistical significance) while PTx has no effect on $Ca_V1.3$ currents in absence of MC4R ($Ca_V1.3$ = −23.63 ± 9.27 pA/pF $n = 7$ and $Ca_V1.3$ + PTx = −20.02 ± 9.43 pA/pF $n = 6$, n.s. *t*-Test), while ChTx failed to do it (Fig. 5). Thus, the MC4R constitutive activity inhibits $Ca_V1.3$ currents in a $G_{i/o}$-dependent pathway.

Since the most commonly reported pathway mediating MC4R signaling involves G_s protein activation and cAMP increase, we used another experimental strategy to test if MC4R constitutive activity can inhibit $Ca_V1.3$ in a G_s/cAMP-independent manner. In particular, we used a MC4R mutant (named MC4R-R18C) that has been shown to couple to G_s under agonist-induced receptor activation but fails to couple to G_s in basal conditions (Srinivasan et al., 2004). As expected, we found that HEK293 cells transfected with the wild-type MC4R display an increase of basal cAMP levels, as compared to untransfected cells, and that the cAMP increment is impaired by chronic exposure to AgRP (Fig. 6A).

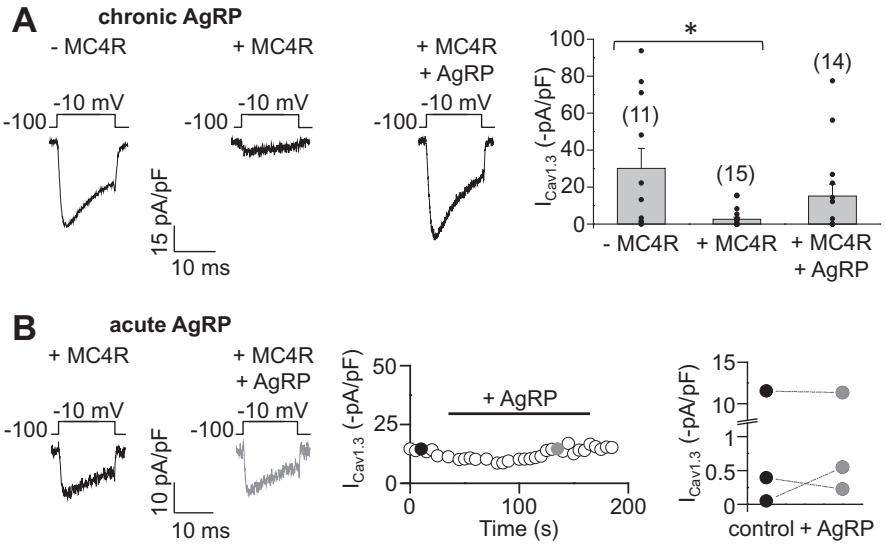

Fig. 4. $Ca_V1.3$ inhibition by MC4R constitutive activity requires a long time to be reverted. (A) MC4R co-expression inhibitory effect on $Ca_V1.3$ currents is partially occluded by chronic incubation with AgRP. Representative traces and average values of $Ca_V1.3$ currents recorded from HEK293 cells co-expressing (+MC4R) or not (−MC4R) MC4R and pre-incubated (+AgRP) or not with 100 nM AgRP during 44 h before the recording (*indicates statistical significant differences among groups, Kruskal–Wallis test and Dunn's multiple comparisons post-test, $p < 0.05$). (B) MC4R co-expression inhibitory effect on $Ca_V1.3$ currents is not modified by acute application of AgRP. The panel displays representative traces and time course of a $Ca_V1.3$ current from transfected HEK293 cells before and after the application of 100 nM AgRP and a graph with the current values for 3 independent cells.

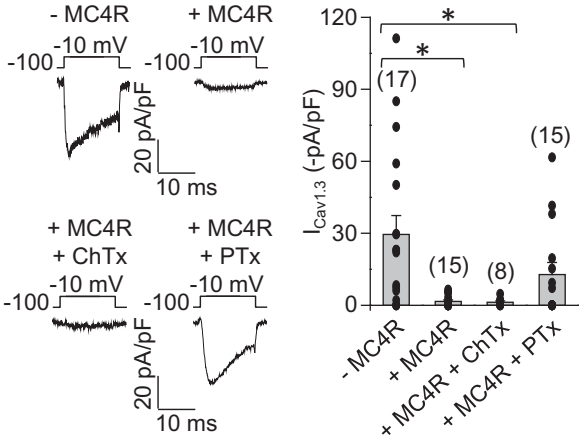

Fig. 5. MC4R constitutive activity requires $G_{i/o}$ protein to inhibit $Ca_V1.3$. Representative traces and average values of $Ca_V1.3$ currents in HEK293 cells without (−MC4R) or with (+MC4R) the co-expression of MC4R in a 1 MC4R/$Ca_V1.3$ molar ratio without or with the 44 h pre-incubation of 500 ng/ml cholera toxin (ChTx) or 500 ng/ml pertussis toxin (PTx). Calcium currents were activated by a square step at −10 mV from a holding potential of −100 mV. (*indicates statistical significant difference from – MC4R condition by a Kruskal–Wallis and Dunn's multiple comparisons post-test, $p < 0.05$).

As previously reported, we confirmed that MC4R-R18C expression in HEK293 cells fails to increase basal cAMP levels, and also found that AgRP failed to affect cAMP levels in MC4R-R18C-expressing HEK293 cells (Fig. 6A). Importantly, transfected MC4R-R18C was functional in our experimental condition since we checked its ability to inhibit $Ca_V2.2$, to the same extent than wild-type MC4R, when it was activated by the synthetic agonist MTII (Fig. 6B). In this scenario, we evaluated if MC4R-R18C co-expression chronically inhibits $Ca_V1.3$ currents in a similar fashion as seen for the wild-type MC4R, and found nominal $Ca_V1.3$ currents in cells co-expressing MC4R or MC4R-R18C (Fig. 6C). Thus, we found that a mutant MC4R unable to couple to G_s in basal conditions chronically reduces $Ca_V1.3$ current as wild type MC4R, confirming that MC4R constitutive activity inhibits $Ca_V1.3$ by a G_s-independent mechanism.

MC4R constitutive activity also targets $Ca_V2.1$ by a $G_{i/o}$-dependent pathway

We found indications that MC4R constitutive activity also inhibits $Ca_V2.1$ current in both amygdaloid cultured neurons and transfected HEK293 cells. Thus, we inquired about the intracellular pathway mediating such inhibitory effect. As seen for the $Ca_V1.3$, we confirmed that MC4R significantly reduced $Ca_V2.1$ currents and found that $Ca_V2.1$ currents in the presence of MC4R have a tendency to increase when cells are pre-incubated with PTx but data groups do not reach statistical difference ($Ca_V2.1$ = −26.89 ± 10.04 pA/pF n = 15, MC4R/$Ca_V2.1$ = −1.20 ± 0.78 pA/pF n = 14, p = 0.0206 vs $Ca_V2.1$ and MC4R/$Ca_V2.1$ + PTx = −13.07 ± 12.00 pA/pF n = 4, ns vs $Ca_V2.1$). On the other hand, the inhibition was unaffected by ChTx ($Ca_V2.1$ = −24.90 ± 8.50 pA/pF n = 10, MC4R/$Ca_V2.1$–1.51 ± 1.01 pA/pF n = 9, p = 0.0358 vs $Ca_V2.1$ and MC4R/$Ca_V2.1$ + ChTx = −4.05 ± 2.69 pA/pF n = 12, n.s. vs MC4R/$Ca_V2.1$) suggesting that the same pathway is shared by these two Ca_V subtypes targeted by MC4R basal activity.

DISCUSSION

Here, we show that MC4R constitutive activity chronically inhibits specific subtypes of neuronal Ca_V. We found that L-type currents are reduced by MC4R co-expression and that this effect is partially occluded by AgRP, an endogenous MC4R inverse agonist. We observed this effect in both isolated L-type Ca_V subtypes, $Ca_V1.2$ and $Ca_V1.3$, and in cultured neurons from the amygdala, a brain area with high MC4R expression. We found a similar effect on P/Q-type ($Ca_V2.1$) currents, while N-type ($Ca_V2.2$) is unaffected by this mechanism. We consider that $Ca_V2.1$ effect is out of the scope of this manuscript, and we expect to complete a study looking at the differential effect of MC4R on synapses governed

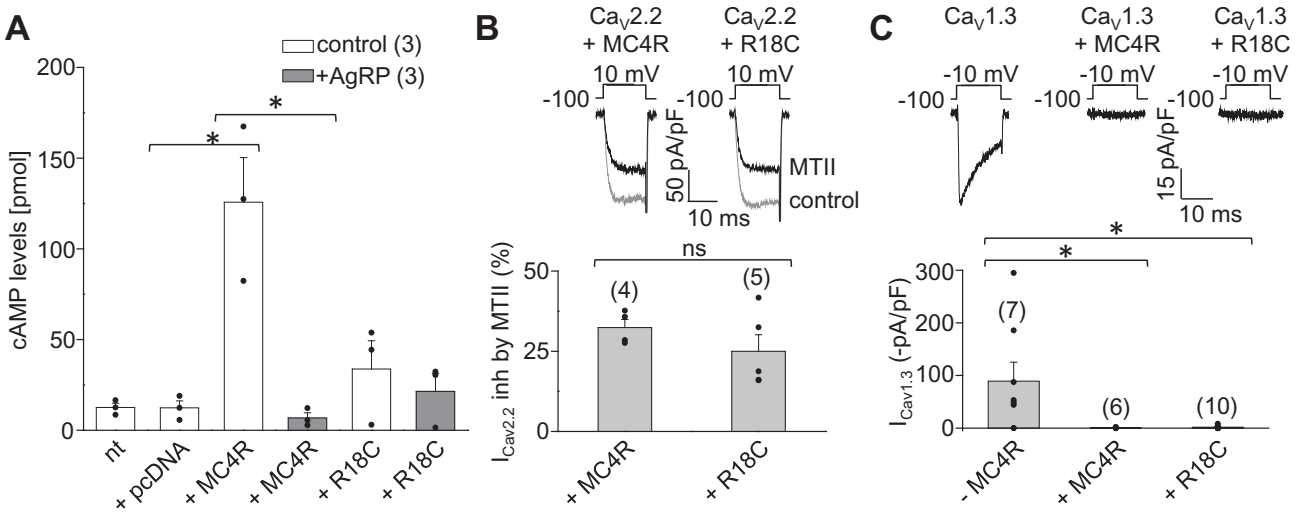

Fig. 6. MC4R constitutive activity inhibits $Ca_V1.3$ independently of cAMP increased levels. (A) cAMP levels are unchanged by MC4R-R18C expression. cAMP levels measured in transfected HEK293 cells transfected or not (nt) with empty plasmid (+pcDNA), MC4R (+MC4R) or mutant MC4R (+R18C) in presence (+AgRP, gray bars) or not (control, white bars) of 100 nM AgRP (*indicates statistical significant difference among groups by Kruskal–Wallis and Dunn's multiple comparisons post-test, $p < 0.05$). (B) MC4R-R18C is functional on HEK293 cells. Representative traces and average values of $Ca_V2.2$ current inhibition by application of MC4R agonist, 250 nM MTII, in HEK293 cells transfected with $Ca_V2.2$, its auxiliary subunits and MC4R (+MC4R) or mutant MC4R (+R18C). (ns = no differences between groups, Student *t*-test, $p < 0.05$). (C) MC4R-R18C constitutive activity inhibits $Ca_V1.3$ current. Representative traces and average values of $Ca_V1.3$ current in HEK293 cells transfected with $Ca_V1.3$, its auxiliary subunits only (−MC4R) or plus MC4R (+MC4R) or mutant MC4R (+R18C). (*indicates statistical significant difference among groups by Kruskal–Wallis and a Dunn's multiple comparison post-test, $p < 0.05$).

by $Ca_V2.2$ and $Ca_V2.1$. Overall, our present results led us to suggest that specific Ca_V subtypes currents are impaired on neurons expressing MC4R and they are, at least partially, de-inhibited in presence of AgRP.

MC4R is the only known GPCR with an endogenous inverse agonist. Several research articles indicated that Agouti and AgRP reduce MC4R constitutive activity, and we have used AgRP as a tool to determine the participation of basal activity on the Ca_V activity reduction by MC4R co-expression. As we indicated in result sections and showed in Figs. 1 and 4, AgRP seems to occlude the inhibitory effect of MC4R basal activity in recombinant channels (Fig. 4) and native neuronal currents (Fig. 1). In the case of recombinant $Ca_V1.3$ in HEK cells, the statistical analysis does not allow us to conclude about the full recover of current to control values. The difficulty to establish the occlusion of MC4R constitutive activity by the inverse agonist could be due that AgRP has a biased effect and only is 100% effective to occlude the effect of MC4R constitutive activity to activate G_s pathway. This possibility is consistent with data presented in Fig. 6A demonstrating a large reduction in AMPc levels by AgRP pre-incubation. This premise is also coherent with the fact that several references testing the effect of AgRP as a MC4R inverse agonist only assessed AMPc levels as a read out of the Gs pathway (Chai et al., 2003; Blondet et al., 2004; Kim et al., 2008; Ersoy et al., 2012). Moreover, in neuronal cultures, the increase in nifedipine sensitive native current by AgRP pre-incubation displays a tendency but does not reach statistical significance. Here, we have to consider also, the natural variations in channels and MC4R expression levels in neurons in the context of an experiment based on independent groups.

MC4R is a GPCR expressed on several brain nuclei controlling the homeostasis of the organism. This particular receptor displays agonist-evoked and constitutive activities, and it can couple to different G proteins (Chai et al., 2006; Newman et al., 2006; Buch et al., 2009; Breit et al., 2011). In a previous work, we showed that MC4R activation by a synthetic agonist, MTII, inhibits N-type Ca_V ($Ca_V2.2$) through a G_s-mediated signaling pathway. Now, we found that MC4R constitutive activity inhibits L-type currents by a G_s-independent but $G_{i/o}$-dependent mechanism, adding new evidence to the already proposed promiscuity of this GPCR (Newman et al., 2006; Breit et al., 2011). Our data also suggest that P/Q-type Ca_V is inhibited by a $G_{i/o}$ protein signaling pathway. G_s intracellular cascade activation is one of the most reported effects of the MC4R constitutive activity (Srinivasan et al., 2004; Tao, 2014). Here we confirmed such notion by measuring cAMP accumulation in HEK293 cells expressing MC4R, but we found that this G_s/cAMP-dependent pathway is not necessary to activate the cascade that inhibits L- and P/Q-type Ca_V. Moreover, we assayed an MC4R mutant, MC4R-R18C, that lacks the ability to couple to G_s under MC4R constitutive activity mode, and observed that it still inhibits L-type currents on basal conditions. The pathway we found as responsible for the basal inhibition of Ca_V currents implicates $G_{i/o}$ activity but also another unidentified G protein and/or a G protein-independent pathway seems to be involved since PTx does not fully recovered the current to control levels. Taking our results together, we propose that agonist-dependent and basal MC4R modes of activity differentially recruit G_s and $G_{i/o}$ to impact on different Ca_V subtypes. Other neuronal conductances are also affected by MC4R activity. Inwardly rectifying potassium channels

can be oppositely regulated by α-MSH (inhibition) and AgRP (activation) in a G protein-independent pathway (Ghamari-Langroudi et al., 2015). But also K_{ATP} channel can be activated by α-MSH after the activation of a cAMP/PKA-dependent cascade (Sohn et al., 2013). Conjointly, the MC4R biased mechanisms that regulate different Ca_V subtypes (showed in our previous and actual work) in addition with the G protein-dependent and -independent mechanism described by other authors to modulate K channels demonstrate the promiscuity of MC4R to activate different intracellular cascades to control neuron activity. In this sense, the final cellular read out after α-MSH/AgRP binding will depend on the neuronal context.

Our observation that chronic MC4R constitutive activity impacts on L-type Ca_V may have important physiological implications. We found that AgRP could occlude this inhibition in both a cell line and cultured neurons. This inhibition couldn't be removed by acute AgRP application, instead, 44-h incubation were necessary to rescue the L-type calcium currents. Consequently we hypothesize that the recovery of L-type current may involve a *de novo* synthesis/trafficking of the Ca_V. Importantly, we propose that the MC4R constitutive activity-induced inhibition of L-type Ca_V depends on a post-translational mechanism and that it is not due to a reduction in the Ca_V gene expression, since it is observed in HEK293 cells in which the gene expression of the Ca_V depends on the constitutive promoters of the plasmids. Therefore, this inhibitory mechanism could involve changes in Ca_V translocation, such as internalization from the plasma membrane, or a decrease in the traffic from the endoplasmic reticulum to the plasma membrane, with the possibility of Ca_V degradation increase on both cases. In this regard, we have recently shown that the constitutive activity of another GPCR, the ghrelin receptor, inhibits pre-synaptic Ca_V by decreasing Ca_V density on the plasma membrane (Lopez Soto et al., 2015). Then, a similar mechanism could mediate the inhibition of L-type Ca_V by MC4R constitutive activity.

As MC4R is expressed at different nucleus of the brain controlling diverse physiological functions (as memory (Machado et al., 2010), food intake (Balthasar et al., 2005), depression (Chaki and Okuyama, 2005)) it is reasonable that the mechanisms of action in neurons are diverse. Our results could explain why administration of the L-type Ca_V blocker, nifedipine, occludes the obesity in mice overexpressing the peptide agouti (Kim et al., 1996), an analog to AgRP (Chai et al., 2003). Here, we show that AgRP increases c-Fos levels in neurons suggesting that the blockage of MC4R constitutive activity activates neurons at the transcriptional level, and we propose that this effect could be due to the presence of more L-type Ca_V in the cell membrane due to occlusion of the chronic inhibitory effect of MC4R. This observation was unexpected since previous reports showed that agonist-induced MC4R activation increased neuronal activity by enhancing transcription (Thiele et al., 1998; Benoit et al., 2000). Since calcium entry through L-type Ca_V controls gene expression in neurons, a plausible explanation is that the AgRP-mediated augmentation in gene expression could be due to the dis-inhibition of L-type currents. More experiments are needed to conclude in this regard, but we could hypothesize that MC4R expressing neurons in the amygdala will have less response to depolarization and calcium influx than neurons without MC4R expression; and that this situation could be modified by persistent presence of AgRP. This reasoning could lead to the following statement: when agouti protein is constitutive expressed (Kim et al., 1996) L-type Ca_V are available in the membrane and the gene transcription dependent on calcium influx could activate food intake, causing obesity and, in absence of agouti proteins, L-type Ca_V are inhibited by MC4R constitutive activity (or by nifedipine on (Kim et al., 1996) report) and obesity can be avoided.

Previous reports have shown that MC4R agonists induce depolarization (Liu et al., 2003) and increase firing in neurons of the paraventricular nucleus of the hypothalamus (Ghamari-Langroudi et al., 2010), another region with high MC4R expression. Thus, our data are opposite since L-type Ca_V would be activated after depolarization. One possible explanation is that acute agonist-induced activation of MC4R has a distinct role from the chronic MC4R constitutive activity. This is in agreement with our data showing that Ca_V subtypes are differentially inhibited by the two modes of activation and targeted by different signaling cascades. One of the main differences among the impact of basal and acute modulation of Ca_V is the dependency of the current inhibition with the amount of MC4R expressed, as we found in controlled experiments in HEK293 cells (Fig. 2). These finding would contribute to explain the intermediate phenotype of MC4R +/− mice between wild type and MC4R −/− mice (Huszar et al., 1997), suggesting that the amount of MC4R is crucial to its precise functionality since it is uncommon that a hemizygote displays an intermediate phenotype.

CONCLUSION

MC4R constitutive activity inhibits L-type calcium currents through a $G_{i/o}$-dependent and G_s-independent mechanism. This effect is Ca_V subtype specific since it was also seen for P/Q-type calcium currents but does not affect N-type calcium currents. This mechanism could have important consequences *in vivo* since when MC4R constitutive activity is occluded by AgRP, neurons seem to be more susceptible to activate gene transcription (as shown by increased c-Fos activation in cultured neurons) suggesting that AgRP de-inhibits L-type Ca_V to be available in the plasma membrane to activate the calcium influx-dependent transcription of genes involved on food intake.

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