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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 test the hypothesis that MC4R constitutive activity can affect Ca_V, with focus on the channel subtypes that are able to 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. © **2017 Published by Elsevier Ltd on behalf of IBRO.**

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). Importantly, MC4R signaling exclusively in the PVN and

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

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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 test 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 Gi/o inhibitor, pertussis

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