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# Melanocortin 4 receptor activation induces brain-derived neurotrophic factor expression in rat astrocytes through cyclic AMP – Protein kinase A pathway

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ABSTRACT

Melanocortin 4 receptors (MC4R) are mainly expressed in the brain. We previously showed that the anti-inflammatory action of α-melanocyte-stimulating hormone (α-MSH) in rat hypothalamus and in cultured astrocytes involved MC4R activation. However, MC4R mechanisms of action remain undetermined. Since brain-derived neurotrophic factor (BDNF) may be mediating MC4R hypothalamic anorexigenic actions, we determined melanocortin effects on BDNF expression in rat cultured astrocytes and certain mechanisms involved in MC4R signaling. α-MSH and its analogue NDP-MSH, induced production of cAMP in astrocytes. This effect was completely blocked by the MC4R antagonist, HS024. We found that NDP-MSH increased BDNF mRNA and protein levels in astrocytes. The effect of NDP-MSH on BDNF expression was abolished by the adenylate cyclase inhibitor SQ22536, and decreased by the PKA inhibitor Rp-cAMP. Since melanocortins are immunomodulators, we investigated their actions with bacterial lipopolysaccharide (LPS) and interferon-γ (IFN-γ) stimulus. Although both α-MSH and LPS + IFN-γ increased cAMP responding element binding protein (CREB) activation, LPS + IFN-γ did not modify BDNF expression. On the other hand, α-MSH did not modify basal or LPS + IFN-γ-induced nuclear factor-κB activation. Our results show for the first time that MC4R activation in astrocytes induces BDNF expression through cAMP-PKA-CREB pathway without involving NF-κB.



## 1. Introduction

Melanocortins (α-, β- and γ-melanocyte-stimulating hormones and adrenocorticotropic hormone) are peptides derived from pro-opiomelanocortin proteolytic cleavage. This system has five receptors (MC1R to MC5R), and two endogenous antagonists (agouti and agouti related peptide). Melanocortins have potent anti-inflammatory, anti-pyretic, anorexigenic and neuroregenerative effects in the brain (Bertolini et al., 2009; Catania et al., 2010). Of the five known MCRs, MC3R and MC4R are the predominant subtypes expressed in the brain, where MC4R distribution is much broader than MC3R distribution (Mountjoy et al., 1994). MC3R is proposed to function as an inhibitory auto-receptor in neurons and is involved in energy homeostasis (Chen et al., 2000). Among the many functions of MC4R, it mediates antipyretic (Sinha et al., 2004) and neuroprotective (Giuliani et al., 2006) actions of α-MSH, and reverses amnesia induced by interleukin-1β (Gonzalez et al., 2009). Targeted disruption of MC4R gene causes obesity-diabetes syndrome (Huszar et al., 1997) and mutations in MC4R gene are associated with severe early-onset obesity (Tao, 2010). MC4R activation was also shown to regulate food intake by inducing the release of brain-derived neurotrophic factor (BDNF) in the hypothalamus (Xu et al., 2003), suggesting that BDNF is a downstream mediator of MC4R action. An analogue of adrenocorticotrophin able to activate all MCRs increased BDNF mRNA levels in rat glial cell cultures (Shadrina et al., 2001), and after cerebral ischemia (Dmitrieva et al., 2010). We previously reported that α-MSH through MC4R reduced hypothalamic expression of inducible nitric oxide synthase and cyclooxygenase-2 induced by bacterial lipopolysaccharide (LPS) in male rats (Caruso et al., 2004). We also demonstrated that α-MSH decreases the release of nitric oxide and prostaglandins induced by LPS and interferon-γ (IFN-γ) in astrocytes, again through

*Abbreviations:* α-MSH, α-melanocyte-stimulating hormone; AC, adenylate cyclase; BDNF, brain-derived neurotrophic factor; cAMP, cyclic AMP; CRE, cAMP responsive element; CREB, cAMP responsive element binding protein; db-cAMP, $N^6$,2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate sodium; HPRT, Hypoxanthine–guanine phosphoribosyltransferase; FBS, fetal bovine serum; MEM-S, supplemented MEM; IκB, nuclear factor κB inhibitor; IFN, interferon; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase; MCR, melanocortin receptor; NDP-MSH, [Nle(4),D-phe(7)]melanocyte-stimulating hormone; NF-κB, nuclear factor κB; PKA, protein kinase A; Rp-cAMP, Rp-adenosine 3′,5′-cyclic monophosphorothioate triethylammonium; TNF, tumor necrosis factor.

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MC4R activation, thereby reducing the inflammatory response and preventing LPS and IFN-γ-induced apoptosis in these cells (Caruso et al., 2007). Recently, we also showed that melanocortins attenuate tumor necrosis factor-α (TNF-α) expression induced by LPS + IFN-γ in cultured hypothalamic neurons that express MC4R (Caruso et al., 2010). However, the mechanisms involved in melanocortins actions are still poorly understood.

All MCRs are G protein coupled receptors that activate adenylate cyclase (AC) leading to cyclic AMP (cAMP) production which, in turn, can activate protein kinase A (PKA) (Lasaga et al., 2008). PKA involvement in melanocortin actions was examined in THP-1 cells (Yoon et al., 2003), in melanoma cells (Ao et al., 1998), and in adrenal cells (Roy et al., 2011). PKA activation *in vivo* rescues the obesity syndrome of agouti lethal yellow mice (Czyzyk et al., 2008). However, until now PKA involvement in melanocortin actions in brain cells has not been investigated. PKA can phosphorylate and activate the cAMP responsive element (CRE) binding protein (CREB), which then acts within the nucleus as a transcription factor. CREB is involved in several physiological processes in the brain by upregulating the expression of several genes (Haus-Seuffert and Meisterernst, 2000), such as BDNF (Tao et al., 1998). CREB is activated by α-MSH in neurons of the hypothalamic paraventricular nucleus (Sarkar et al., 2002), in neurons of the solitary nucleus (Sutton et al., 2005) and in hypothalamic cultured neurons (Caruso et al. 2010). α-MSH can also modulate the activation of the nuclear factor-κB (NF-κB) (Manna and Aggarwal, 1998). NF-κB is a transcription factor that regulates the inflammatory response and is held in the cytoplasm in an inactive state by its inhibitor (IκB) (Li and Verma, 2002). Once phosphorylated, IκB dissociates, and free NF-κB translocates to the nucleus where it activates target gene transcription. We previously showed that cultured astrocytes express MC4R but not MC3R (Caruso et al., 2007), and Selkirk et al. (2007) also indicated that MC4R is the only MCR expressed in these cells. Therefore, astrocytes are a suitable model to investigate MC4R-mediated actions. Although some reports show signaling pathways activated by over-expressed MCRs in cell lines, mechanisms and mediators of melanocortin effects in glial cells have not yet been elucidated. In this study, we show that MC4R activation increases BDNF expression in astrocytes in a cAMP-PKA-CREB dependent fashion but independent of NF-κB activation.

## 2. Materials and methods

### 2.1. Reagents

α-MSH and [Nle(4),D-phe(7)]melanocyte-stimulating hormone (NDP-MSH) were obtained from Bachem California (CA, USA). HS024 was purchased from Tocris Bioscience (MO, EE.UU). LPS (*Escherichia coli*, serotype O127:B8) was purchased from Sigma–Aldrich Corporation (MO, USA). IFN-γ was purchased from Boehringer Ingelheim, Argentina. DMEM/F-12, antibiotics and glutamine were purchased from Invitrogen Life Technologies (CA, USA). Biotinylated donkey anti-mouse and anti-rabbit antibodies were obtained from Chemicon (Millipore, MA, USA). Anti-NF-κB p65 was obtained from BD Biosciences (CA, USA) and anti-IκBα was purchased from Cell Signaling Technology (MA, USA). Anti-BDNF was provided by Santa Cruz Biotechnologies (CA, USA). BDNF and hypoxanthine–guanine phosphoribosyltransferase (HPRT) primers were purchased from Invitrogen Life Technologies (CA, USA). Rp-adenosine 3′,5′-cyclic monophosphorothioate triethylammonium (Rp-cAMP), $N^6$,2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate sodium (db-cAMP) were purchased from Sigma–Aldrich Corporation, and SQ22536 was from Calbiochem. All other media and supplements were obtained from Sigma–Aldrich Corporation, unless otherwise specified.

### 2.2. Cell culture

Astrocytes were prepared from rat cerebral tissue of 1- to 2-day-old postnatal Wistar rat pups as described previously (Caruso et al., 2007). Cells were maintained in DMEM/F-12 medium containing 10% fetal bovine serum (FBS, PAA laboratories GmBH, Pasching, Austria), 50 μg/ml streptomycin, 50 U penicillin in 75 $cm^2$ poly-L-lysine coated culture flasks at 37 °C in 5% $CO_2$. Cell culture medium was changed twice a week. After 11–14 days, astrocytes were separated from microglia and oligodendrocytes by shaking overnight at 200 rpm. Cells were trypsinized, subcultured, and after 2–3 days of stabilization, incubated with the drugs in MEM containing 2% FBS, 2 mM L-glutamine, 50 μg/ml streptomycin, and 50 U penicillin (MEM-S). Cultures were routinely more than 95% pure astrocytes, as assessed by glial fibrillary acidic protein immunostaining. Experimental procedures were carried out with approval of the ethical committee guidelines of the School of Medicine of the University of Buenos Aires, which are in accordance with National Institute of Health Guidelines for the Care and Use of Laboratory Animals.

### 2.3. Intracellular cAMP determination

Astrocytes (40,000 cells/well) were plated in 96-well plates and after 3 days of stabilization, the medium was replaced with MEM-S containing the drugs to be tested. After 20 min incubation at 37 °C, the medium was discarded and the cells were lysed with 100 μl 0.1 N HCl, centrifuged and supernatant was frozen at −70 °C. Supernatants were lyophilized and subsequently resuspended in assay buffer. Levels of intracellular cAMP were determined by an enzyme immunoassay kit (Assay Designs, MI, USA) following the manufacturer's instructions.

### 2.4. Reverse transcriptase-real time polymerase chain reaction (RT-qPCR)

Total RNA from cultured astrocytes ($1 \times 10^6$ cells) was extracted using TRIZOL reagent (Invitrogen Life Technologies) according to the manufacturer's protocol. About 2 or 5 μg of total RNA was treated with 1 U RQ1 RNase free-DNase (Promega Corporation, WI, USA) at 37 °C for 10 min. cDNA synthesis was carried out using 0.1 μg oligo-dT primers (Invitrogen) plus SuperScript II (Invitrogen) for 2 h at 37 °C or Improm-II (Promega) for 1 h at 42 °C with 3 mM MgCl. Products of the RT reaction were amplified using specific primers (400 nM for BDNF and 450 nM for HPRT) and SYBR Green Master Mix (Applied Biosystem) on a StepOne™ Real-Time PCR System (Applied Biosystem). Synthetic oligonucleotides used for qPCR were **BDNF** forward 5′-GATGAGGACCAGAAGGTTCG-3′, **BDNF** reverse 5′-TCCAGCAGAAAGAGCAGAGG-3′, **HPRT** forward 5′-CTCATGG ACTGATTATGGACAGGAC-3′, **HPRT** reverse 5′-GCAGGTCAGCAAAG AACTTATAGCC-3′. PCR conditions were denaturation at 95 °C for 1 min, followed by 40 cycles of 95 °C for 1 min and 68 °C for 1 min. The PCR product specificity was verified by a melting curve analysis. No-RT controls were performed by omitting addition of the reverse transcriptase enzyme, and no-template controls were performed by addition of nuclease free water instead of cDNA. Levels of BDNF expression were normalized to the endogenous reference gene HPRT using the relative quantitative method (ΔΔCt) (Livak and Schmittgen, 2001).

### 2.5. Western blot analysis

Astrocytes ($1 \times 10^6$ cells) were lysed and proteins obtained as described previously (Caruso et al., 2007). Protein concentration of samples was determined by Bradford assay. About 40 μg of protein was size-fractionated in a SDS–polyacrylamide gel and then,

electrotransferred to polyvinylidene difluoride membrane. Blots were blocked for 2 h in 5% nonfat dry milk–Tris–buffered saline (TBS)–0.1% Tween 20 and incubated overnight with the appropriate primary antibody diluted (anti-NF-κ 1:400, anti-IκBα 1:2000, anti-BDNF 1:1000) in 5% milk–TBS–0.1% Tween 20 at 4 °C. After washing the blots, they were incubated with the respective biotinylated secondary antibody for 1 h. Immunoreactivity was detected by enhanced chemiluminescence (ECL plus, Amersham Biosciences, GE Healthcare). Results were normalized to the internal control β-actin and values were expressed as increments relative to respective controls using SCION Image software.

### 2.6. Preparation of nuclear extracts

Astrocytes ($5 \times 10^6$ cells) were treated with the drugs for 1 h and then, nuclear extracts were prepared. Cells were harvested by adding 0.05% trypsin–EDTA (Gibco), washed twice with ice-cold phosphate buffered saline and lysed into buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 2 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1% nonidet p-40, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, 5 μg/ml leupeptin, 500 μM sodium orthovanadate, 1 mM sodium fluoride) for 10 min on ice and centrifuged at 14,000 rpm for 5 min. The supernatant containing the cytoplasmic proteins was removed and stored at −70 °C until use. The pellet was resuspended in buffer B (20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, 5 μg/ml leupeptin, 500 μM sodium orthovanadate and 1 mM sodium fluoride). After incubation on ice for 30 min lysates were centrifuged at 14,000 rpm for 15 min and the supernatants containing the nuclear proteins were stored at −70 °C until use.

### 2.7. Electrophoretic mobility shift assay

Nuclear protein extracts from treated astrocytes were used for the electrophoretic mobility shift assay. This assay was carried out essentially as previously described (Caruso et al., 2010). CREB consensus sequence was end-labeled with [α-$^{32}$P]dATP. The $^{32}$P end-labeled CRE oligonucleotide (0.5 ng) was incubated with nuclear extracts (5 μg) in a total volume of 20 μl containing 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 μg poly d(I-C), 1 μg BSA and 10% glycerol. After 20 min incubation at room temperature, the binding reactions were analyzed by electrophoresis on a 5% polyacrylamide gel in 0.25X Tris-boric acid–EDTA buffer and visualized by autoradiography. Competition experiments were performed by incubating nuclear proteins with the appropriate unlabeled oligonucleotide for 10 min prior to the addition of the labeled probe.

### 2.8. Statistical analysis

Data were expressed as mean ± SEM and were analyzed by one sample *t* test, Student *t* test, or one-way analysis of variance (ANOVA) followed by Dunnet or Bonferroni Multiple Comparisons Test. Differences with a $p < 0.05$ were considered statistically significant. All experiments were performed at least twice.

## 3. Results

### 3.1. MC4R activation stimulates cAMP production in rat astrocytes

Since MC4Rs are G-protein coupled receptors that positively regulate AC, we determined the effect of MC4R agonists on cAMP production in cultured rat astrocytes. α-MSH (1 μM) and the α-MSH analogue, NDP-MSH (1 μM), increased cAMP intracellular levels, whereas forskolin, an AC activator used as a positive control, was a much stronger cAMP inducing agent (Fig. 1A). Since astrocytes only expressed MC4R (Caruso et al., 2007; Selkirk et al., 2007), we examined the effect of the selective MC4R antagonist, HS024, on the stimulatory effect of NDP-MSH on cAMP production. HS024 completely abolished the increase in cAMP levels elicited by NDP-MSH (Fig. 1B) whereas HS024 by itself decreased cAMP production (Fig. 1B).

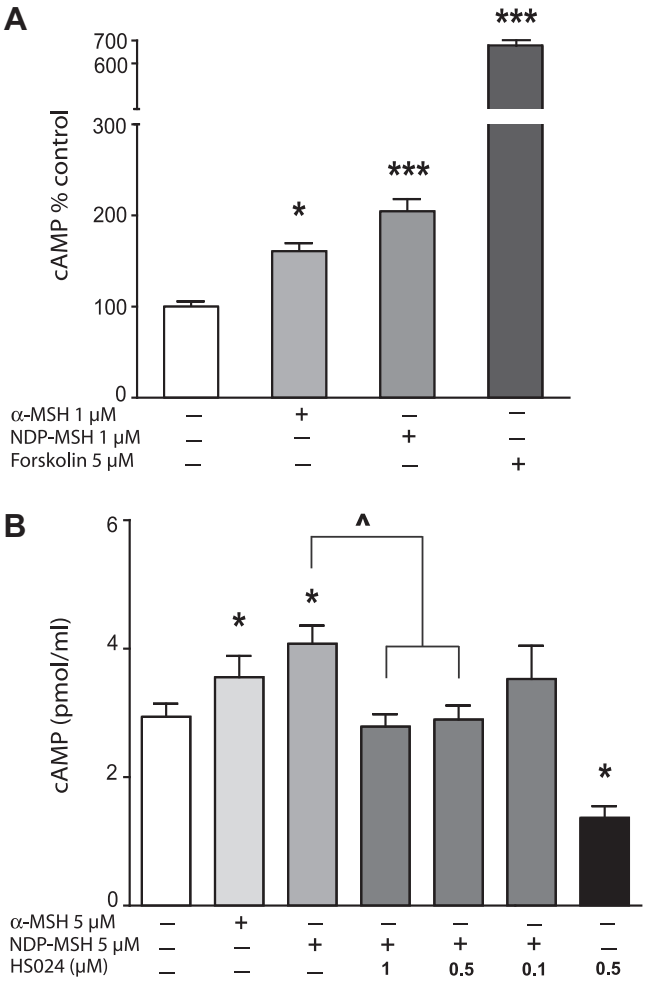


**Fig. 1.** Effect of MC4R agonists and antagonist on intracellular cAMP levels in astrocytes. Astrocytes were treated with the drugs for 20 min. Intracellular cAMP levels in cell lysates were determined by an enzyme-linked immunoassay. Values are the mean ± SEM of 4–5 determinations for each group of 2 independent experiments and were analyzed by one way Anova. $^{*}p < 0.05$ and $^{***}p < 0.001$ vs. control group and $^{\wedge}p < 0.05$ vs. NDP-MSH.

### 3.2. MC4R activation increases BDNF expression in rat astrocytes

Since BDNF mediates MC4R anorexigenic effects in the hypothalamus (Xu et al., 2003) and BDNF transcription is controlled by cAMP in astrocytes (Juric et al., 2008), we tested whether BDNF expression was induced by MC4R activation in astrocytes. BDNF mRNA levels were increased by all tested concentrations of NDP-MSH after 1 and 3 h of incubation (Fig. 2A). BDNF protein levels were increased at 24 h by NDP-MSH treatment (Fig. 2B), although BDNF mRNA returned to basal levels at this time (data not shown).

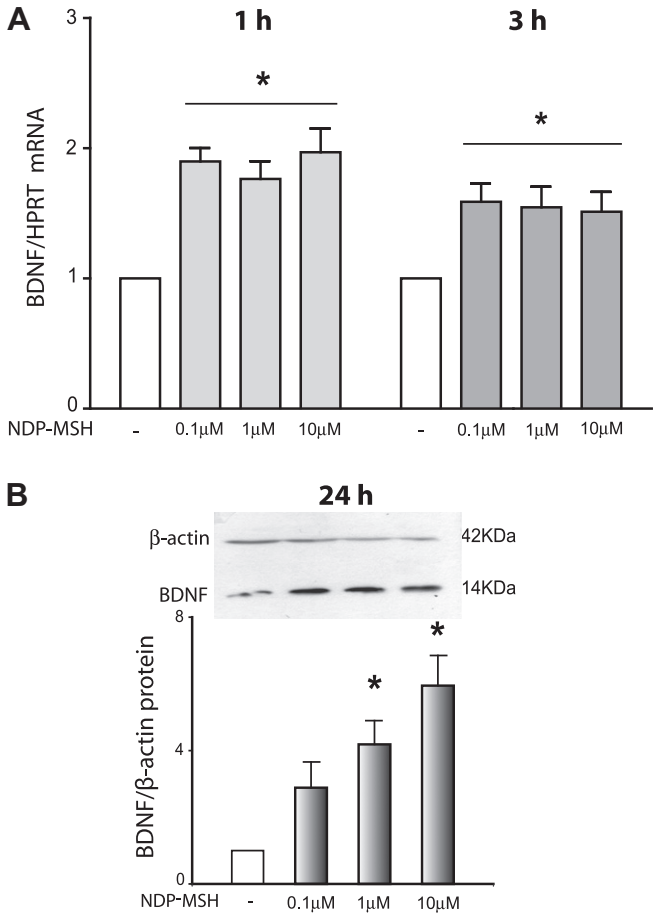


**Fig. 2.** MC4R activation induces BDNF expression in astrocytes. Astrocytes were treated with NDP-MSH (0.1, 1, 10 μM). (A) RNA was isolated from astrocytes after treatment for 1 and 3 h and processed for real time RT-PCR quantification. Values represent the BDNF/HPRT ratio compared to control group of 3 independent experiments and were analyzed by one sample *t* test. $^{*}p < 0.05$ vs. control group. (B) Cell lysates from astrocytes treated for 24 h were subjected to Western blot. Membranes were probed with antibodies against BDNF and β-actin. Values represent the BDNF/β-actin ratio compared to control group of 3 independent experiments and were analyzed by one sample *t* test. $^{*}p < 0.05$ vs. control group.

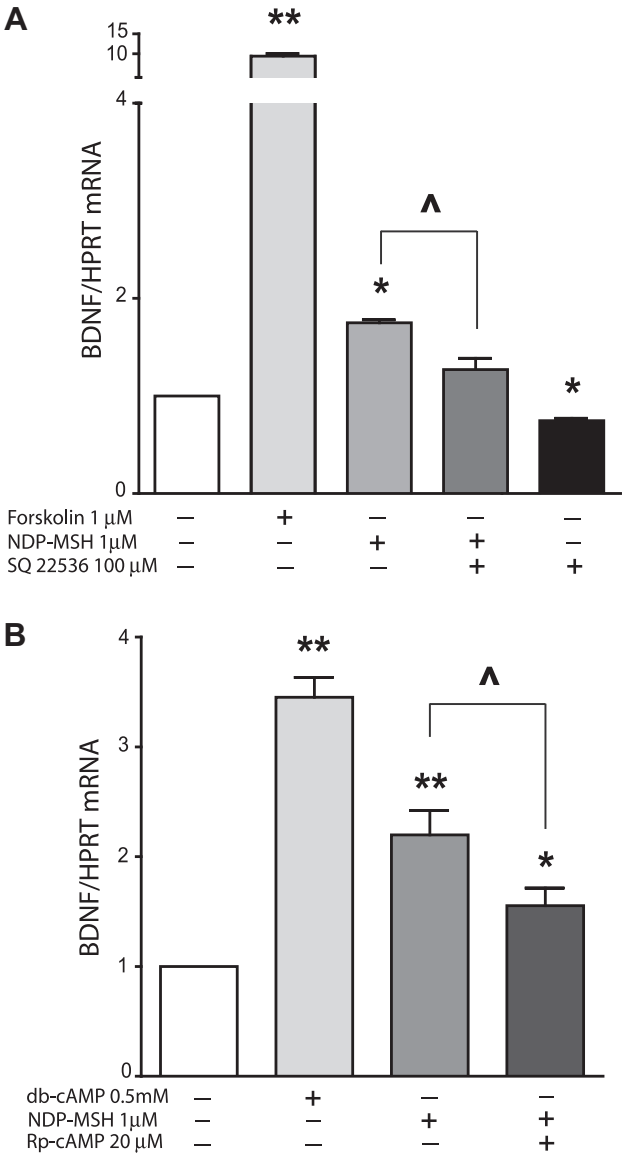


**Fig. 3.** cAMP and PKA are involved in MC4R agonist-induced BDNF expression in astrocytes. Astrocytes were preincubated with the inhibitor of AC (SQ22536, A) or of PKA (Rp-cAMP, B) for 15 min before they were treated with NDP-MSH for 1 h. RNA was isolated from astrocytes after treatment and processed for real time RT-PCR quantification. Values represent the BDNF/HPRT ratio compared to control group of 3 independent experiments and were analyzed by one sample *t* test. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control group and $^{\wedge}p < 0.05$ vs. NDP-MSH.

### 3.3. cAMP-PKA pathway mediates the increase in BDNF expression induced by MC4R activation in astrocytes

We examined cAMP involvement in the increase in BDNF expression at 1 h induced by MC4R activation. Forskolin 1 μM, a potent AC activator, induced a higher increase in BDNF compared to 1 μM NDP-MSH (Fig. 3A). Accordingly, the AC inhibitor, SQ22536 (100 μM), prevented the increase in BDNF expression induced by NDP-MSH. Interestingly, SQ22536 also decreased basal BDNF mRNA levels. We then tested the effect of db-cAMP, a cAMP analogue that activates PKA. db-cAMP 0.5 mM, enhanced BDNF expression at 1 h (Fig. 3B). The cAMP competitive PKA inhibitor, Rp-cAMP 20 μM, significantly inhibited NDP-MSH-induced BDNF expression. However, this inhibition was partial, since BDNF levels were still higher than in control group (Fig. 3B). All together, these data indicate that cAMP production and PKA activation are involved in MC4R-induced BDNF expression in astrocytes.

### 3.4. MC4R agonist induces CREB but not NF-κB activation

Previous results from our laboratory indicate that MC4R activation by α-MSH reduces nitric oxide and prostaglandin production induced by LPS (1 μg/ml) + IFN-γ (50 ng/ml) in astrocytes (Caruso et al., 2007), indicating that melanocortins modulate inflammation. Therefore, we investigated MC4R mechanism of action in the presence of this pro-inflammatory stimulus. We found that LPS + IFN-γ significantly reduced cAMP content whereas, as shown before, α-MSH increased cAMP levels in astrocytes (Fig. 4A). However, α-MSH blocked LPS + IFN-γ-induced decrease of intracellular cAMP levels (Fig. 4A). Also, MC4R antagonist HS024 blocked this effect (Fig. 4A). Since cAMP production ultimately leads to phosphorylation of CREB, we evaluated CREB activation in nuclear protein extracts from astrocytes. α-MSH induced CREB activation, as also did LPS + IFN-γ, although both treatments together had no additive effects (Fig. 4B). Since LPS + IFN-γ induce CREB activation, and BDNF transcription is regulated by CREB (Tao et al., 1998), we investigated whether this inflammatory stimulus induced BDNF expression. However, LPS + IFN-γ failed to modify BDNF mRNA levels at 1 and 3 h (Fig. 5). Moreover, co-incubation of LPS + IFN-γ

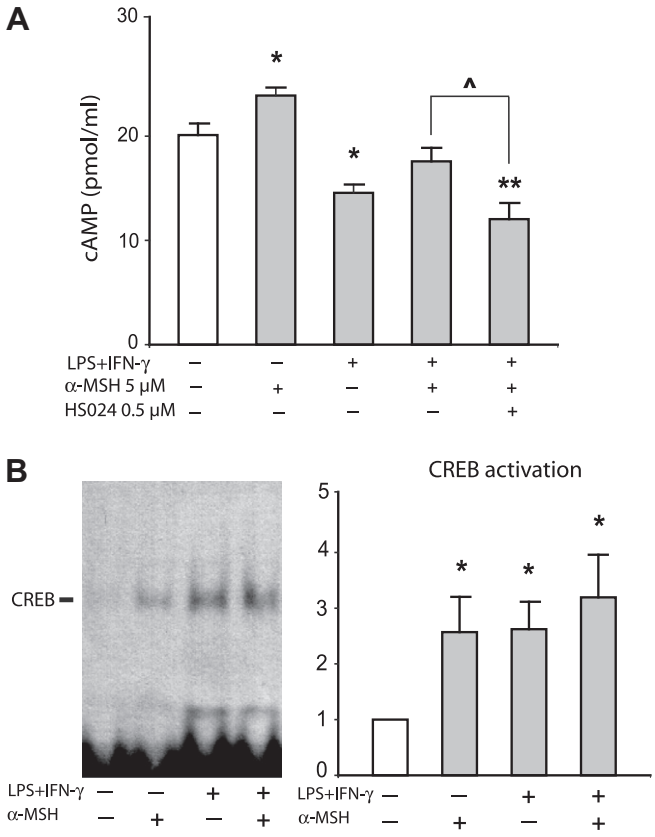


**Fig. 4.** MC4R agonist and LPS + IFN-γ induce CREB activation in astrocytes. (A) Astrocytes were treated with LPS (1 μg/ml) + IFN-γ (50 ng/ml) with or without α-MSH (5 μM) or HS024 for 20 min. Intracellular cAMP levels in cell lysates were determined by an enzyme-linked immunoassay. Values are the mean ± SEM of 3 independent experiments and were analyzed by one way Anova. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control group and $\hat{}p < 0.05$ vs. LPS + IFN-γ + α-MSH. (B) Semiquantification of CREB activation. Astrocytes were treated with LPS + IFN-γ with or without α-MSH 5 μM for 1 h and then, they were harvested for isolation of nuclear proteins. CRE/CREB complexes were detected in nuclear proteins. Values are the mean ± SEM of 2 independent experiments and were analyzed by one sample *t* test. $^{*}p < 0.05$ vs. control group.

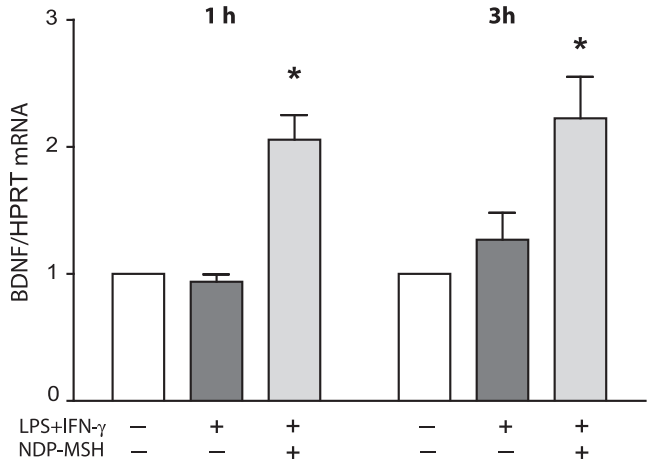


**Fig. 5.** MC4R activation induces BDNF expression in LPS + IFN-γ-activated astrocytes. Astrocytes were treated with LPS (1 μg/ml) + IFN-γ (50 ng/ml) with or without NDP-MSH 5 μM. RNA was isolated from astrocytes after treatment for 1 and 3 h and processed for real time RT-PCR quantification. Values represent the BDNF/HPRT ratio compared to control group of 3 independent experiments and were analyzed by one sample *t* test. $^{*}p < 0.05$ vs. control group.

with NDP-MSH increased BDNF expression (Fig. 5), indicating that BDNF is induced by MC4R activation in astrocytes in an inflammatory milieu. These data indicate that MC4R activation induces cAMP-CREB signaling in astrocytes.

α-MSH was shown to decrease activation of NF-κB in different cell lines (Manna and Aggarwal, 1998). NF-κB was also reported to regulate BDNF transcription in glioma cells (Yoon et al., 2008). Therefore, it was of interest for us to determine the effect of α-MSH on NF-κB activation. For that purpose we determined the translocation to the nucleus of the NF-κB p65 subunit that is known to be responsible for activating transcription. The MC4R agonist *per se* did not significantly modify either NF-κB translocation or cytoplasmic levels of IκB α (Fig. 6). As expected, LPS + IFN-γ significantly increased NF-κB translocation to the nucleus and decreased cytoplasmic levels of IκBα (Fig. 6). However, MC4R activation neither prevented NF-κB translocation nor modified IκBα degradation induced by LPS + IFN-γ (Fig. 6). These results indicate that NF-κB activation is not affected by melanocortins in astrocytes.

## 4. Discussion

MCRs are key players in the melanocortin system and are promising targets for pharmacological intervention in a variety of conditions. MC4R is known to participate in behavioral as well as sexual functions, food intake, inflammation, and neuroprotection (Tao, 2010). However, the mechanisms and mediators involved in the action of MC4R are not fully understood. Our present results show for the first time that activation of MC4R by melanocortins leads to increased BDNF expression in rat astrocytes. We also demonstrated that MC4R-induced BDNF expression is cAMP-PKA dependent and that MC4R agonists induce CREB activation without involving NF-κB.

Selkirk et al. (2007) showed that MC4R agonists elicited different responses in cultured astrocytes compared to cell lines overexpressing MC4R. Therefore, it is important to be cautious when interpreting results from cell lines. Since astrocytes only express MC4R (Selkirk et al., 2007; Caruso et al., 2007), they provide a good model to investigate MC4R-mediated effects. Activation of MCRs stimulates AC (Lasaga et al., 2008). Accordingly, our data show that both MCR agonists (NDP-MSH and α-MSH) increase cAMP production in rat astrocytes. The fact that HS024, a selective MC4R antagonist, completely blocked the increase in cAMP levels induced by NDP-MSH further supports the fact that MC4R is the only MCR present in astrocytes. Interestingly, HS024 by itself decreased cAMP production in astrocytes, and it has been reported that another MC4R antagonist, HS014, diminished *per se* cAMP production in melanocytes (Spencer and Schallreuter, 2009), suggesting that MC4R could be activated in basal conditions as it has been shown before (Haskell-Luevano and Monck, 2001).

We demonstrate here that MC4R activation increases BDNF mRNA and protein expression in rat astrocytes. BDNF, a neurotrophin widely distributed in the CNS, is released in response to MC4R agonist administration in the hypothalamus *in vivo* (Xu et al., 2003). *In vivo* administration of an anti-BDNF antibody also blocks the anorexigenic effect of a MC4R agonist (Nicholson et al., 2007). Our data show increased BDNF expression in astrocytes, therefore it is possible that astrocyte-derived BDNF could contribute to the anorexigenic effect of melanocortins in the hypothalamus, further indicating that astrocytes are very important for the maintenance of brain homeostasis. In addition, BDNF released from astrocytes can exert neuroprotective action in oligodendrocytes (McTigue et al., 1998), and in neurons as it is well documented (Binder and Scharfman, 2004). Since BDNF receptors are present in astrocytes (Climent et al., 2000) this neurotrophin could also have autocrine

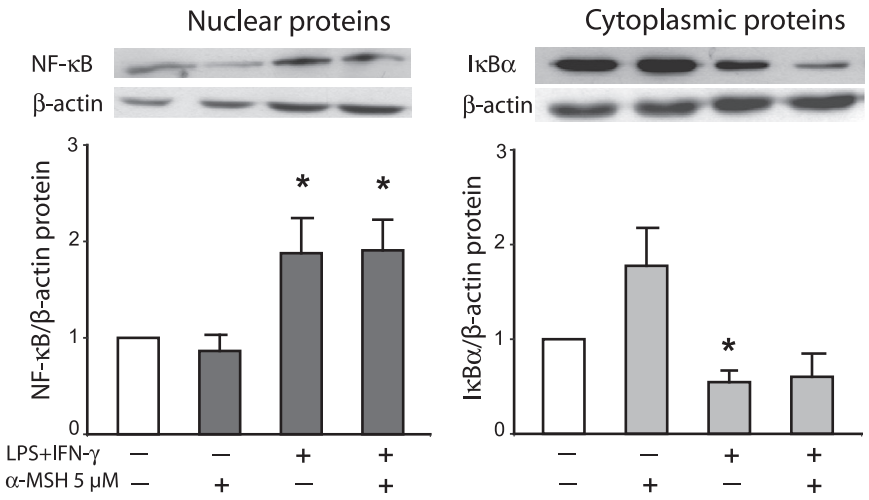


**Fig. 6.** MC4R agonist fails to modify NF-κB activation. Semiquantification of nuclear NF-κB and cytoplasmic IκBα protein levels in astrocytes treated for 1 h. Nuclear cell extracts were prepared and then, nuclear and cytoplasmic proteins were subjected to Western blot. Membranes with nuclear proteins were probed with anti-NF-κB and anti-β-actin antibodies, and cytoplasmic proteins were probed with anti-IκBα and anti-β-actin antibodies. Data are expressed as mean ± SEM of NF-κB/β-actin or IκBα/β-actin ratio of 6 independent experiments and were analyzed by one sample *t* test. $^{*}p < 0.05$ vs. control group.

effects. Although it is well established that BDNF promotes neuron survival, neurite growth, and synaptic plasticity, BDNF effects in astrocytes are less well known. Moreover, BDNF could be a mediator of the anti-apoptotic effects of melanocortins that we and others have previously described (Caruso et al., 2007; Chai et al., 2006).

We also demonstrate that the cAMP-PKA pathway is involved in the increased BDNF expression induced by MC4R activation since it was inhibited by an AC inhibitor (SQ 22536) and a PKA inhibitor (Rp-cAMP). The importance of cAMP-PKA pathway was shown in agouti mice where PKA catalytic subunit was deregulated and therefore PKA activity rescues from obesity syndrome (Czyzyk et al., 2008). Accordingly, *in vivo* administration of MC4R agonist induces activation of the mitogen activated protein kinase (MAPK) ERK dependent on PKA activation (Sutton et al., 2005). However, in CHO-K1 cells over-expressing human MC4R PKA is not involved in ERK activation (Vongs et al., 2004). Moreover, AC is not involved in activating ERK by cells over-expressing MC2R (Roy et al., 2011) and in MC5R over-expressing cells neither cAMP production nor PKA activation is involved in ERK activation (Rodrigues et al., 2009). Evidently, results on MCR signaling vary considerably depending on the cellular context (Breit et al., 2011). Our present results show that cAMP-PKA pathway is important in MC4R signaling in astrocytes. In addition, AC seems to play a major role in MC4R signaling since its inhibition completely blocked BDNF expression induced by NDP-MSH, whereas PKA inhibition partially diminished BDNF mRNA levels induced by NDP-MSH. Therefore, other signaling pathways parallel to PKA might be activated by MC4R as it has been described (Breit et al., 2011). We also showed that α-MSH activates CREB, confirming the activation of cAMP-PKA-CREB pathway by MC4R activation. The finding that the AC inhibitor significantly reduced basal BDNF mRNA levels together with the finding that HS024 decreases basal cAMP, strongly suggest that MC4R may be constitutively active in cultured astrocytes. Indeed, MC4R was reported to have constitutive activity when expressed in cell lines (Haskell-Luevano and Monck, 2001) but until now no evidence of constitutive activity in primary cultured cells had been reported.

We previously demonstrated that melanocortins modulate inflammation induced by LPS + IFN-γ through MC4R in astrocytes. In the present study, LPS + IFN-γ treatment diminishes cAMP levels whereas it increases CREB activation in astrocytes, suggesting that this latter action is not cAMP-dependent. LPS-decreased cAMP levels could be due to inhibition of AC expression (Risoe et al., 2007), or to increased phosphodiesterase activity, leading to degradation of cAMP (Okonogi et al., 1991). Hence, CREB activation by LPS + IFN-γ could be achieved by mechanisms other than cAMP-induced PKA activation, such as ERK and/or p38 phosphorylation (Buzas et al., 2002). We also showed that activation of MC4R abolishes LPS + IFN-γ inhibitory action on cAMP accumulation, an effect that is blocked, in turn, by antagonizing MC4R, suggesting that MC4R activation counteracts LPS + IFN-γ effects.

CREB activation is involved in several physiological processes in the CNS (Lonze and Ginty, 2002). Phosphorylation of CREB is sufficient for target gene activation in response to cAMP and calcium signals, but gene activation in response to other stimuli requires additional factors that synergize with CREB (Lonze and Ginty, 2002). This could explain the differential activation of cellular genes by CREB in response to various signals. Here, we show that NDP-MSH and LPS + IFN-γ increase binding activity of CREB in astrocytes and we have observed the same effect for both stimuli in hypothalamic neurons (Caruso et al., 2010). However, we also show that LPS + IFN-γ do not modify BDNF expression whereas MC4R activation increases BDNF expression in astrocytes, indicating that although CREB is equally activated by these stimuli, it leads to different cellular responses. In a similar way, it was reported that cAMP signals completely activate CREB whereas non-cAMP signals only phosphorylate CREB but do not induce CREB mediated transcription (Mayr et al., 2001). Also, it was recently shown that CREB is fully activated by increasing cAMP, leading to production of anti-inflammatory IL-10 whereas LPS phosphorylates CREB but does not lead to CRE-genes transcription (Avni et al., 2010). Moreover, in the liver LPS increases CRE binding activity but decreases CRE mediated reporter activity (Zhang et al., 2004). Therefore, CREB activation by LPS + IFN-γ may have no effect or inhibit transcription of CRE-genes, but CREB activation by NDP-MSH via cAMP-PKA pathway induces transcription of anti-inflammatory and neuroprotective genes, such as BDNF shown here or Bcl-2 as we previously reported (Caruso et al., 2007).

Although BDNF participation in inflammation has not been extensively studied, BDNF was reported to inhibit activation of astrocytes and microglia induced by ischemia and to reduce expression of inducible nitric oxide synthase in rat hippocampus (Kiprianova et al., 1999). Moreover, it was recently shown that

BDNF is produced by glial cells following stroke in both monkeys (Sato et al., 2009) and rats (Béjot et al., 2011), and that intranasal administration of BDNF reduces TNF-α and increases interleukin-10 mRNA levels in a model of ischemia (Jiang et al., 2011). Therefore, BDNF could be a mediator of anti-inflammatory effects of melanocortins exerted through MC4R in astrocytes. In addition, in multiple sclerosis lesions BDNF is primarily present in immune cells and reactive astrocytes (De Santi et al., 2011), indicating that astrocytic BDNF could play a beneficial role in neurodegenerative disorders.

MC4R activation does not affect basal or LPS + IFN-γ-induced NF-κB activation in astrocytes. Similarly, we recently showed that α-MSH failed to modify NF-κB activation induced by LPS + IFN-γ in hypothalamic neurons (Caruso et al., 2010). Despite the fact that melanocortins inhibit NF-κB activation in cell lines (Manna and Aggarwal, 1998) α-MSH action on NF-κB activation in glial cell is still controversial. α-MSH in human glioma cells reduced (Ichiyama et al., 1999) whereas in H4 glioma cells did not modify NF-κB activation (Sarkar et al., 2003). Moreover, NF-κB action in brain is more complex than previously thought since it was recently reported that astroglial inhibition of NF-κB does not ameliorate the onset and progression of a mouse model for a neurodegenerative disease, amyotrophic lateral sclerosis (Crosio et al., 2011). Therefore, modulation of NF-κB activation is extremely variable depending on the stimulus and the cell type. NF-κB was suggested to regulate BDNF expression in C6 glioma cells (Yoon et al., 2008) and in neurons (Kairisalo et al., 2009). Our present results show that melanocortins induce BDNF expression in a NF-κB independent manner, suggesting that different stimulus can activate different mechanisms to induce the same effect. Since activation of MCRs can inhibit (Yoon et al., 2003) or potentiate (Zhang et al., 2009), signaling pathways activated by other receptors we cannot exclude the possibility that MC4R may modulate other signaling pathways induced by LPS by a different mechanism.

In conclusion, our work shows that MC4R activation in astrocytes increases BDNF expression through a cAMP-PKA dependent pathway that leads to CREB activation but does not involve NF-κB. Our results add valuable knowledge concerning MC4R signaling and suggest that BDNF could be a mediator of MC4R-mediated effects. The elucidation of MC4R signaling pathways involved in melanocortins effects in the brain could help to develop future strategies for the treatment of brain disorders.

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