Molecular and Cellular Endocrinology 411 (2015) 28–37

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Melanocortin 4 receptor activates ERK-cFos pathway to increase brain-derived neurotrophic factor expression in rat astrocytes and hypothalamus

D. Ramírez, J. Saba, L. Carniglia, D. Durand, M. Lasaga, C. Caruso *

INBIOMED UBA-CONICET, School of Medicine, University of Buenos Aires, Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 15 December 2014
Received in revised form 9 April 2015
Accepted 9 April 2015
Available online 16 April 2015

Keywords:
MC4R
BDNF
ERK
cFos
Astrocytes
Hypothalamus

ABSTRACT

Melanocortins are neuropeptides with well recognized anti-inflammatory and anti-apoptotic effects in the brain. Of the five melanocortin receptors (MCR), MC4R is abundantly expressed in the brain and is the only MCR present in astrocytes. We have previously shown that MC4R activation by the α-melanocyte stimulating hormone (α-MSH) analog, NDP-MSH, increased brain-derived neurotrophic factor (BDNF) expression through the classic cAMP–Protein kinase A–cAMP responsive element binding protein pathway in rat astrocytes. Now, we examined the participation of the mitogen activated protein kinases pathway in MC4R signaling. Rat cultured astrocytes treated with NDP-MSH 1 μM for 1 h showed increased BDNF expression. Inhibition of extracellular signal-regulated kinase (ERK) and ribosomal p90 S6 kinase (RSK), an ERK substrate, but not of p38 or JNK, prevented the increase in BDNF expression induced by NDP-MSH. Activation of MC4R increased cFos expression, a target of both ERK and RSK. ERK activation by MC4R involves cAMP, phosphoinositide-3 kinase (PI3K) and the non receptor tyrosine kinase, Src. Both PI3K and Src inhibition abolished NDP-MSH-induced BDNF expression. Moreover, we found that intraperitoneal injection of α-MSH induces BDNF and MC4R expression and activates ERK and cFos in male rat hypothalamus. Our results show for the first time that MC4R-induced BDNF expression in astrocytes involves ERK-RSK-cFos pathway which is dependent on PI3K and Src, and that melanocortins induce BDNF expression and ERK-cFos activation in rat hypothalamus.

1. Introduction

Melanocortins are produced from pro-opiomelanocortin precursor cleavage. These neuropeptides include α-, β- and γ-melanocyte-stimulating hormones (MSH) and adrenocorticotropic hormone. Melanocortins are known to exert protective actions in the brain (Caruso et al., 2012b; Catania, 2008). There are five known melanocortin receptors (MCRs) designated MC1R through MC5R. Of the five known MCRs, **MC4R** is the only subtype expressed in rat astrocytes (Caruso et al., 2007; Selkirk et al., 2007). MC4R mediates antipyretic (Sinha et al., 2004), neuroprotective (Giuliani et al., 2006), and anorexigenic (Marsh et al., 1999) actions of α-MSH. MC4R activation reduces amnesia induced by interleukin-1β (Gonzalez et al., 2009) and it also improves cognitive performance in animals with impaired memory consolidation caused by interleukin-1β (Machado et al., 2010). We have previously shown that MC4R mediates the anti-inflammatory action of melanocortins in cultured astrocytes and in the hypothalamus (Caruso et al., 2004, 2007). More recently, we demonstrated that MC4R activation in astrocytes leads to increased brain-derived neurotrophic factor (BDNF) expression (Caruso et al., 2012a). BDNF is a neurotrophic factor essential for neuronal development, survival, and synaptic plasticity (Lewin and Barde, 1996) and has been proposed to participate in the anorexigenic effect of melanocortins in hypothalamus (Xu et al., 2003).

MC4R is a G-protein coupled receptor (GPCR) that through a classic signaling pathway activates adenylate cyclase (AC) leading to cAMP synthesis which in turn activates protein kinase A (PKA). Then PKA activates cAMP responsive element binding protein (CREB), which was shown to be activated by α-MSH in neurons (Caruso et al., 2010; Sutton et al., 2005). We recently showed that MC4R activation

Abbreviations: α-MSH, α-melanocyte-stimulating hormone; AC, adenylate cyclase; BDNF, brain-derived neurotrophic factor; CREB, cAMP responsive element binding protein; DAPI, 4′,6-diamido-2-phenylindole dihydrochloride; ERK, extracellular signal-regulated kinase; GFAP, glial fibrillary acidic protein; GPCR, G protein-coupled receptor; HMB, hypothalamic fragment; HPRT, hypoxanthine–guanine phosphoribosyltransferase; MAPK, mitogen activated protein kinase; MCR, melanocortin receptor; NDP-MSH, [Nle(4),D-Phe(7)]melanocyte-stimulating hormone; PKA, protein kinase A; PI3K, phosphoinositide-3 kinase; PDK1, phosphoinositide-3-dependent protein kinase-1; Rp-cAMP, Rp-adenosine 3′,5′-cyclic monophosphorothioate triethylammonium; RSK, ribosomal p90 S6 kinase; RT-qPCR, reverse transcriptase- real time polymerase chain reaction.

* Corresponding author. Instituto de Investigaciones Biomédicas (INBIOMED) UBA-CONICET, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires 1121ABG, Argentina. Tel.: +54 11 5950 9500 ext 2158; fax: +54 11 5950 9612.
E-mail address: ccaruso@fmed.uba.ar (C. Caruso).

http://dx.doi.org/10.1016/j.mce.2015.04.008
0303-7207/

in astrocytes increases BDNF expression in a cAMP-PKA-CREB dependent fashion (Caruso et al., 2012a). However, MC4R can also activate other signaling pathways that can contribute to its biological effects (Breit et al., 2011). Mitogen-activated protein kinases (MAPK) are a family of serine-threonine protein kinases involved in proliferation, migration and stress processes in the cell. MC4Rs activate the MAPK/extracellular signal-regulated kinase (ERK) when overexpressed in GT1-7 cells (Chai et al., 2006; Damm et al., 2012) and in vivo in the solitary nucleus (Sutton et al., 2005). Some reports also show that ERK activation may be either PKA-independent (Chai et al., 2006; Vongs et al., 2004) or dependent (Sutton et al., 2005). Also, it was shown that MC4R inhibits JNK activity when expressed in HEK293 cells (Chai et al., 2009) and inhibits p38 activation induced in a model of Alzheimer's disease (Giuliani et al., 2014). It was also suggested that MC4R may induce p38 activation in dorsal root ganglion (Chu et al., 2012). Therefore, MAPK involvement in MC4R action is dependent on the cell type where it is expressed. The phosphoinositide-3 kinase (PI3K)/Akt pathway is also involved in cell proliferation and survival. When activated, PI3K induces phosphoinositide-3-dependent protein kinase-1 (PDK1) activation by phosphorylation. Then, PDK1 phosphorylates other protein kinases such as Akt and p90 ribosomal protein S6 kinase (RSK) which can also be activated by ERK. Other studies also described that NDP-MSH, an analog of α-MSH, through MC4R or α-MSH through MC5R can induce ERK activation that is mediated by PI3K (Rodrigues et al., 2009; Vongs et al., 2004). However, MC4R signaling within the brain is still incompletely understood.

We previously showed that astrocytes express only MC4R and that its activation induces BDNF expression through cAMP-PKA-CREB pathway in these cells. In the present work we investigated MAPKs involvement in MC4R signaling in astrocytes. We found that ERK is the only MAPK activated by NDP-MSH through PI3K and Src. We showed that ERK-RSK-cFos pathway is activated by MC4R in astrocytes through which BDNF expression is increased. We also showed that ERK-cFos pathway is activated by melanocortins in vivo in rat hypothalamus as well.

2. Methods

2.1. Materials

α-MSH and [Nle(4),D-Phe(7)]melanocyte-stimulating hormone (NDP-MSH) were obtained from Bachem California (CA, USA). 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). Phospho- ERK, phospho-Akt and total Akt antibodies were purchased from Cell Signaling Technology (MA, USA). Total ERK antibody was provided by Santa Cruz Biotechnology (CA, USA). All primers used were purchased from Invitrogen Life Technologies (CA, USA). Rp-adenosine 3′,5′-cyclic monophosphorothioate triethylammonium (Rp-cAMP), Forskolin, GSK233470, BRD7389, PP2 and 1,3-dihydro-1-(1-((4-(6-phenyl-1*H*-imidazo[4,5-g]quinoxalin-7-yl)phenyl) methyl)-4-piperidinyl)-2H-benzimidazol-2-one trifluoroacetate salt hydrate (Akt1/2 inhibitor) were purchased from Sigma-Aldrich Corporation. SQ22536, LY294002, PD98059, SB203580, SP600125 were from Calbiochem. OSU-03012 was purchased from Selleck Chemicals. The concentrations of inhibitors used in this work are higher than the published IC50 concentrations except for OSU03012 and BRD7389, both used at the IC50 concentration. Protein kinase inhibitors can have detrimental effects on cell viability if they are used at high concentrations. Therefore, we tested whether the inhibitors used in this work affected astrocyte viability by MTT assay. On the basis of these results we selected the highest dose of the inhibitor that did not induce cell death of astrocytes (Table 1). All other media and supplements were obtained from Sigma-Aldrich Corporation, unless otherwise specified.

Table 1
Kinase inhibitors used in this study.

Target molecule	Inhibitor name	IC50	Dose used (μM)
MEK	PD98059	2–7 μM	10
p38	SB203580	50 nM	10
JNK	SP600125	40–90 nM	20
PI3K	LY294002	Up to 6.6 μM	20
Akt	Akt inhibitor	58–210 nM	0.5
AC	SQ22536	1.4–20 μM	100
PKA	Rp-cAMP	11 μM	20
PDK1	OSU03012	1–5 μM	1
PDK1	GSK2334470	10 nM	1
Src	PP2	0.73 μM	2
BRD	BRD7389	1 μM	1

2.2. Animals

Wistar rats were housed in a temperature-controlled facility at 25 °C on a 12 h light/12 h dark cycle with access to lab chow and water ad libitum. All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of the School of Medicine of the University of Buenos Aires, which are in line with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

2.3. 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 (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. Cells were trypsinized and subcultured and, after 2–3 days of stabilization, incubated with the drugs in MEM containing 2% fetal bovine serum, 6 mM L-glutamine, 50 μg/ml streptomycin, and 50 U penicillin. Cultures were routinely more than 95% pure astrocytes, as assessed by glial fibrillary acidic protein (GFAP) immunostaining.

2.4. In vivo experiments

Male Wistar rats 2 months old were randomly divided into two groups: (**3h**) rats were injected with α-MSH (0.5 mg/kg) or vehicle (saline) intraperitoneally (ip) once and killed 3 h later by decapitation; (**48h**) rats were injected with α-MSH (0.5 mg/kg) or vehicle ip twice a day for 2 days and killed by decapitation 48 h after the first injection. Then, a hypothalamic fragment containing the paraventricular, arcuate, dorsomedial and ventromedial nucleus (HMB) was dissected and immediately processed. In another set of experiments, animals treated for 48 h with α-MSH were anesthetized with ketamine (75 mg/kg) and xilazine (10 mg/kg) and transcardially perfused with 120 ml of PBS containing heparin followed by 120 ml of PBS–2% picric acid–4% paraformaldehyde. Brains were immediately removed and post-fixed in PBS–4% paraformaldehyde for 1 h. Then, the tissue was cryoprotected in 20% sucrose in PBS solution for 1–2 weeks. Coronal brain sections of 50 μm were cut with a cryostat (Leica Zeiss) following Paxino's rat brain atlas (Paxinos and Watson, 1997) for selection of the hypothalamus sections and the free floating sections were subjected to immunohistochemistry.

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

Total RNA from cultured astrocytes (1×10^6 cells) or from HMB was extracted using QUICK-ZOL reagent (Embiotec, Argentina) according to the manufacturer's protocol. Two micrograms 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 done with 0.2 μg oligo-dT primers (Biodynamics) plus Improm-II (Promega) for 1 h at 42 °C with 3mM MgCl. Products of the RT reaction were amplified using specific primers 400 nM for BDNF and 450 nM for hypoxanthine–guanine phosphoribosyltransferase (HPRT) with SYBR Green Master Mix (Applied Biosystems) as previously described (Caruso et al., 2012a) or 150 nM for BDNF, 200 for MC4R and cFos and 150/200 nM for HPRT with SYBR Select Master Mix (Applied Biosystems) on a StepOne™ Real-Time PCR System (Applied Biosystems). Synthetic oligonucleotides used for qPCR were: **BDNF** forward 5′-GATGAGGACCAGAAGGT TCG-3′, **BDNF** reverse 5′-TCCAGCAGAAAGAGCAGAGG-3′, **HPRT** forward 5′-CTCATGGACTGATTATGGACAGGAC-3′, **HPRT** reverse 5′-GCAGGTCAGCAAAGAACTTATAGCC-3′, **cFos** forward: 5′-TGACAGATAC GCTCCAAGCG-3′, **cFos** reverse: 5′-TGGCAATCTCGGTCTGCA-3′, **MC4R** forward: 5′-CCAACTGCAGCCTTCCAACT-3′, **MC4R** reverse: 5′-GCCCCGAGTGGTTTTTCTC-3′.

The PCR product specificity was verified by a melting curve analysis. Negative controls were performed by omitting addition of the reverse transcriptase enzyme in RT reaction and by addition of nuclease free water instead of cDNA in PCR reaction. Levels of BDNF, MC4R and cFos expression were normalized to the endogenous reference gene HPRT using the relative quantitative method (ΔΔCt) as previously reported (Caruso et al., 2012a).

2.6. Western blot analysis

Astrocytes (1×10^6 cells) or HMB were lysed and proteins were obtained as previously described (Durand et al., 2011). Protein concentration of samples was determined by Bradford assay. 30–40 μg of protein was size-fractionated in a SDS–polyacrylamide gel and then electrotransferred to a polyvinylidene difluoride membrane. Blots were blocked for 1 h in 5% nonfat dry milk–TBS–0.1% Tween 20 and incubated overnight with the appropriate primary antibody. Anti-phosphorylated ERK (pERK, 1:2000) or anti-phosphorylated Akt (pAkt, 1:1000) antibodies were incubated in 5% BSA–TBS–0.1% Tween 20. After being washed, blots were incubated with the respective biotinylated secondary antibody for 1 h. Immunoreactivity was detected by enhanced chemiluminescence (Biolumina, PBL, Argentina). After stripping, the blots were incubated with total ERK (1:2000) or total Akt (1:2000) antibodies respectively diluted in 5% milk–TBS–0.1% Tween 20 at 4 °C. Blots were analyzed using SCION Image software. Data were normalized to total ERK or Akt and values were expressed as increments relative to the respective controls.

2.7. Immunofluorescent identification of cFos in astrocytes

cFos was identified by indirect immunofluorescence staining. After 2 h incubation with NDP-MSH, cells were fixed with 0.5 ml 4% paraformaldehyde–PBS for 30 min at 4 °C. After rinsing with PBS cells were incubated with 10% normal goat serum in PBS with 0.2% Triton X-100 for 60 min. Then, slides were incubated overnight at 4 °C with rabbit anti-cFos antibody (1:1000, Santa Cruz Biotechnologies) in PBS with 0.2% Triton X-100 and 1% normal goat serum. After rinsing, slides were incubated for 1 h with goat anti-rabbit fluorescein-conjugated secondary antibody (1:200, Vector Laboratories Inc.). After washing, slides were mounted with mounting medium for fluorescence (Vectashield, Vector Laboratories Inc.) containing 4′,6-diamido-2-phenylindole dihydrochloride (DAPI) for DNA staining and visualized in a fluorescence microscope (Axiophot, Carl Zeiss, Jena, Germany). Control slides were incubated with normal donkey and goat serum instead of primary antibody as negative controls. cFos immunoreactivity was quantified as pixels using Image J program and shown as fluorescence intensity.

2.8. Immunohistochemistry

Free-floating sections were washed in PBS (pH 7.4) and incubated for 1 h in blocking buffer (10% normal goat and normal donkey serum, and 0.2% Triton X-100 in PBS). Then, sections were incubated for 48–72 h at 4 °C with anti-BDNF antibody (1:1000, AB1779, Millipore), anti-MC4R rabbit antibody (1:150, 10006355 Cayman Chemicals), anti-cFos rabbit antibody (1:1000, sc-546 Santa Cruz Biotechnology), anti-NeuN (1:1000, MAB377, Millipore) and anti-GFAP (1:500, MAB360, Millipore) diluted in 1% normal goat serum, 1% normal donkey serum, 0.2% Triton X-100 in PBS. After washing in PBS, sections were further incubated with secondary antibodies: goat anti-rabbit IgG conjugated with fluorescein (1:200, Vector Laboratories) and donkey anti-mouse IgG conjugated with Cy3 (1:500, Millipore) for 1 h. Finally, sections were mounted onto slides using Vectashield (Vector Laboratories Inc.) as mounting medium. Four to six hypothalamic sections from two rats per group were analyzed. Images were captured using a Nikon Confocal Microscope. BDNF and MC4R immunoreactivity was quantified as pixels using Image J program and shown as fluorescence intensity. cFos-immunoreactive nuclei were counted on every image and the number of immunoreactive nuclei was averaged for each rat.

2.9. Statistical analysis

Data were expressed as mean ± SEM and were analyzed by one sample t test, Student t test, one-way or two-way ANOVA, followed by Bonferroni multiple comparisons test. Differences with a $p < 0.05$ were considered statistically significant. All experiments were performed at least three times.

3. Results

3.1. Involvement of MAPKs pathway in MC4R action in astrocytes

As we previously reported, NDP-MSH (1 μM) increased BDNF mRNA levels in astrocytes (Fig. 1A). In order to evaluate the role of MAPKs in MC4R signaling, we treated astrocytes with inhibitors of p38, JNK and ERK MAPKs and determined the effect of NDP-MSH on BDNF expression. Preincubation with p38 inhibitor SB203580 (10 μM) had no effect on basal or NDP-MSH-induced BDNF mRNA levels (Fig. 1A). The JNK inhibitor SP600125 (20 μM) increased BDNF expression per se (Fig. 1A). SP600125 also increased BDNF expression in the presence of NDP-MSH, this effect being greater than that of NDP-MSH alone (Fig. 1A), suggesting that JNK may have an inhibitory role in BDNF expression in astrocytes. On the contrary, MEK inhibitor PD98059 (10 μM) had no effect on basal BDNF expression although it completely blocked NDP-MSH-induced BDNF expression (Fig. 1A). NDP-MSH increased ERK1/2 phosphorylation in astrocytes and this effect was inhibited by PD98059 (Fig. 1B). RSK was shown to be a substrate and an important effector of ERK-mediated transcriptional regulation (Romeo et al., 2012). Thus, we determined RSK participation by using BRD7389, a novel specific RSK inhibitor (Fomina-Yadlin et al., 2010). BRD7389 (1 μM) blocked NDP-MSH stimulatory effect on BDNF expression although it did not modify BDNF expression by itself (Fig. 1C), indicating that the ERK-RSK pathway is involved in MC4R-mediated effect on BDNF expression in astrocytes.

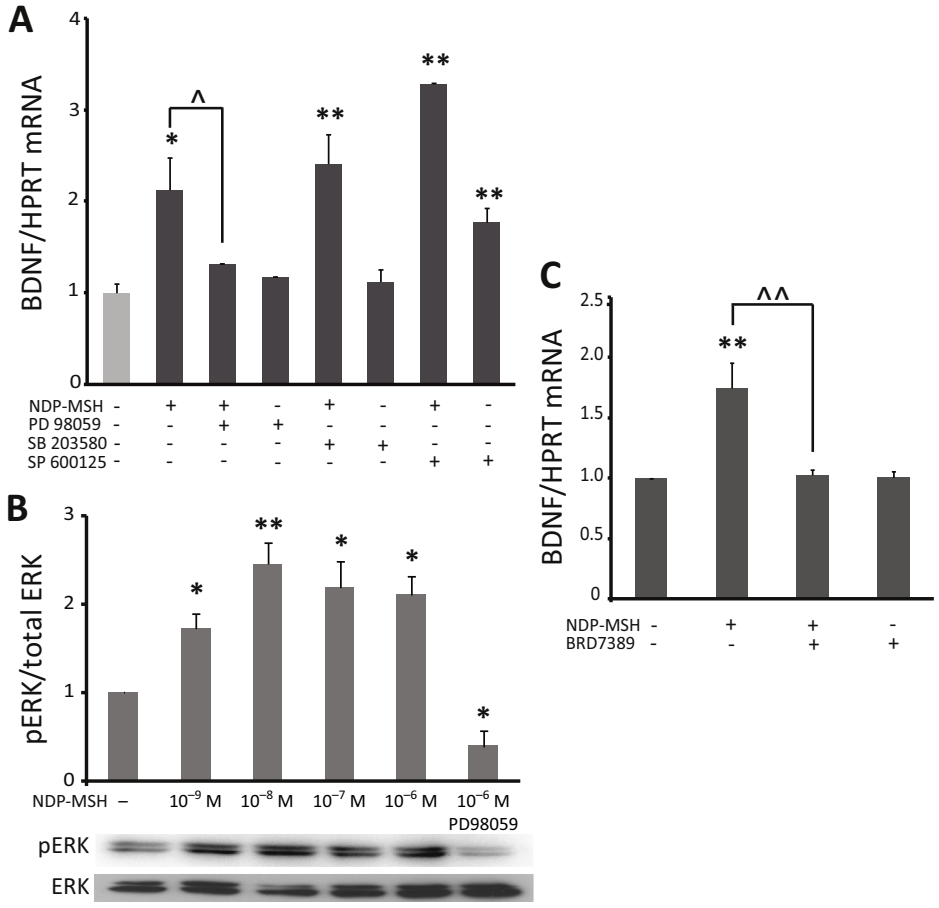

Fig. 1. ERK and RSK mediate the increase in BDNF expression induced by NDP-MSH in astrocytes. (A) Astrocytes were preincubated for 15 min with inhibitors of ERK, p38 and JNK (**PD98059** 10 μM, **SB203580** 10 μM and **SP600125** 20 μM respectively) and then treated with 1 μM **NDP-MSH** for 1 h. Total RNA was extracted and processed for RT-qPCR. Values are the mean of BDNF/HPRT ratio compared to control group of 3–4 independent experiments and were analyzed by two-way ANOVA. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control group and $^{\wedge}p < 0.05$ vs. NDP-MSH. (B) Western blot of cell lysates from astrocytes preincubated with **PD98059** for 15 min and then treated for 30 min with different doses of **NDP-MSH**. Membranes were probed with antibodies against pERK and total ERK. Values represent the pERK/total ERK ratio compared to control group of 4 independent experiments and were analyzed by one sample t test. $^{*}p < 0.05$ vs. control group. (C) Astrocytes were pre-incubated for 15 min with the RSK inhibitor **BRD7389** (1 μM) and then treated with 1 μM **NDP-MSH** for 1 h. Total RNA was extracted and processed for RT-qPCR. Values are the mean of BDNF/HPRT ratio compared to control group of 4 independent experiments and were analyzed by two-way ANOVA. $^{**}p < 0.01$ vs. control group and $^{\wedge\wedge}p < 0.01$ vs. NDP-MSH.

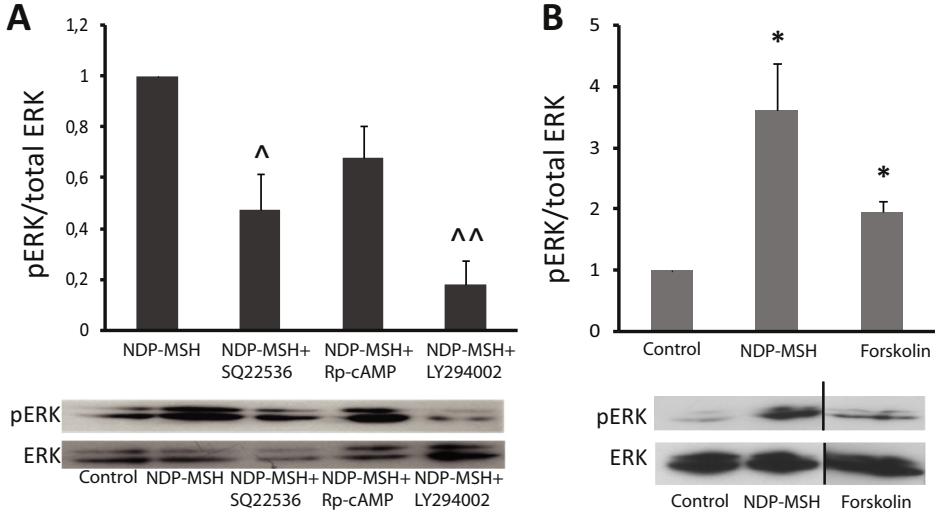

Fig. 2. ERK activation by NDP-MSH in astrocytes is AC and PI3K-dependent. Western blots of cell lysates from astrocytes preincubated with 100 μM **SQ22536**, 20 μM **Rp-cAMP** or 20 μM **LY294002** were treated for 30 min with 1 μM NDP-MSH (A) or astrocytes were treated for 30 min with 1 μM **NDP-MSH** or 10 μM **Forskolin** (B). Membranes were probed with antibodies against pERK and total ERK. Values represent the pERK/total ERK ratio compared to control group of 4 independent experiments and were analyzed by one sample t test. One representative blot of 4 independent experiments is shown where the Forskolin group was run with control and NDP-MSH groups but in non-adjacent lanes. (A) $^{\wedge}p < 0.05$ and $^{\wedge\wedge}p < 0.01$ vs. NDP-MSH. (B) $^{*}\ p < 0.05$ vs. control group.

We previously demonstrated that BDNF expression is induced by MC4R activation through the cAMP-PKA pathway in astrocytes (Caruso et al., 2012a). Therefore, we now tested the involvement of this signaling pathway in ERK1/2 activation. The AC inhibitor SQ22536 (100 μM) reduced ERK1/2 activation induced by NDP-MSH (Fig. 2A). In addition, the AC activator forskolin (1 μM) also induced ERK1/2 phosphorylation (Fig. 2B). PI3K inhibition by LY294002 (20 μM) completely abolished ERK1/2 phosphorylation induced by NDP-MSH (Fig. 2A). Conversely, the PKA inhibitor, Rp-cAMP (20 μM), had no effect on the increase in ERK1/2 phosphorylation induced by NDP-MSH (Fig. 2A). These data indicate that MC4R-induced ERK activation depends on PI3K and partially on cAMP production in astrocytes.

3.2. Participation of PI3K/Akt pathway in MC4R action in astrocytes

In order to investigate PI3K/Akt pathway involvement in MC4R signaling we first treated astrocytes with PI3K inhibitor LY294002 (20 μM) and determined BDNF expression. LY294002 completely abolished the increase in BDNF mRNA levels induced by NDP-MSH and it also reduced basal BDNF mRNA levels (Fig. 3A). PDK1 is a downstream target of PI3K that, in turn, is able to activate Akt and RSK. To test whether PDK1 is involved in NDP-MSH-induced BDNF expression, we treated astrocytes with the PDK1 inhibitors OSU-03012 (Zhu et al., 2004) and the more specific GSK2334470 (Najafov et al., 2011). We found that OSU-03012 (1 μM) had no effect on NDP-MSH stimulation of BDNF expression, nor did GSK2334470 (1 μM) (Fig. 3A). Moreover, an inhibitor of Akt (0.5 μM) did not modify BDNF expression induced by NDP-MSH (Fig. 3A). We also determine the levels of Akt phosphorylation as an indication of its activation and found that NDP-MSH had no effect on Akt phosphorylation (Fig. 3B). These results indicate that MC4R activation involves PI3K but does not activate the classic PI3K/Akt pathway.

Src belongs to the Src family of kinases, a group of non receptor protein kinases. Src integrates and regulates survival signals from tyrosine kinase receptors and activates the PI3K/Akt pathway (Yeatman, 2004) as well as the ERK pathway (Liebmann, 2001). Since ERK1/2 activation by GPCR can be mediated by Src (Ma et al., 2000), and Src can also be activated by PI3K itself (Liebmann, 2001), we tested whether Src was involved in MC4R signaling. Treatment of

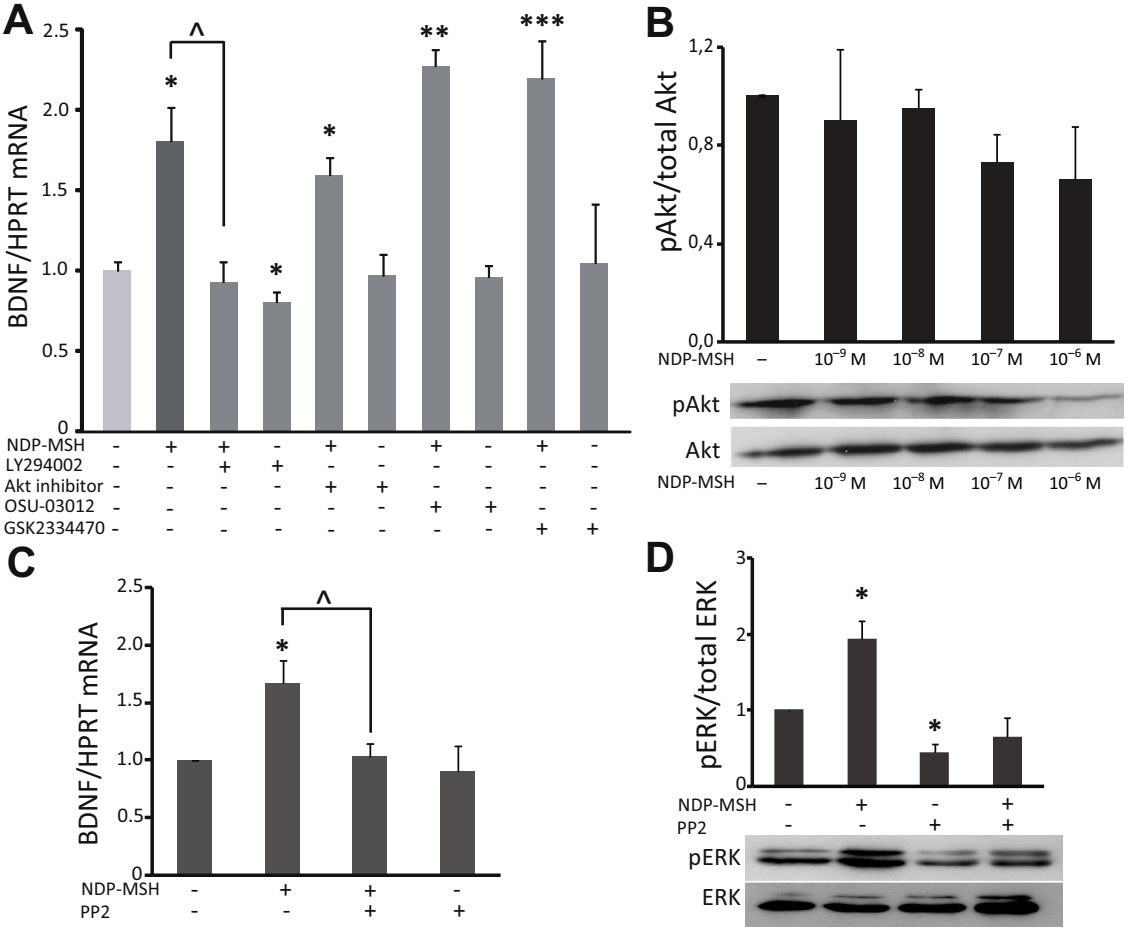

Fig. 3. PI3K and Src participate in ERK activation induced by NDP-MSH. (A) Astrocytes were preincubated for 15 min with 20 μM **LY294002** or 0.5 μM **Akt inhibitor**, or **PDK1 inhibitors** (1 μM **OSU-03012** and 1 μM **GSK2334470**) and then treated with 1 μM **NDP-MSH** for 1 h. Total RNA was extracted and processed for RT-qPCR. Values are the mean of BDNF/HPRT ratio compared to control group of 3 independent experiments and were analyzed by two-way ANOVA. ***$p < 0.001$ **$p < 0.01$ and *$p < 0.05$ vs. control group and ^$p < 0.05$ vs. NDP-MSH. (B) Astrocytes were treated for 30 min with different doses of **NDP-MSH** and then protein extracts were used for detection of pAkt and total Akt by Western blot. Values represent the pAkt/Akt ratio compared to control group of 4 independent experiments and were analyzed by one sample t test. One representative blot of 4 independent experiments is shown. (C) Astrocytes were preincubated with the Src inhibitor **PP2** (2 μM) for 15 min and then treated with 1 μM **NDP-MSH** for 1 h. Total RNA was extracted and processed for RT-qPCR. Values represent the BDNF/HPRT ratio compared to control group of 4 independent experiments and were analyzed by two-way ANOVA. *$p < 0.05$ vs. control group and ^$p < 0.05$ vs. NDP-MSH. (D) Western blot of cell lysates from astrocytes preincubated with **PP2** (2 μM) for 15 min and then treated with 1 μM **NDP-MSH** for 30 min. Membranes were probed with antibodies against pERK and total ERK. Values represent the pERK/total ERK ratio compared to control group of 4 independent experiments and were analyzed by one sample t test. One representative blot of 4 independent experiments is shown. *$p < 0.05$ vs. control group.

astrocytes with Src inhibitor PP2 (2 μM) abolished the increase in BDNF expression induced by NDP-MSH (Fig. 3C). Inhibition of Src decreased basal as well as NDP-MSH-induced increase in pERK1/2 levels (Fig. 3D), indicating that Src is involved in MC4R signaling upstream of ERK in astrocytes.

3.3. MC4R activation induces cFos expression in astrocytes

Immediate early gene *cfos* encodes a transcription factor that together with a member of the Jun family proteins forms the AP1 complex. AP1 regulates transcription of many genes involved in cell survival and death (Perez-Cadahia et al., 2011). cFos activation can be induced by multiple pathways such as ERK-RSK. Transcription of *cfos* as a result of ERK activation can be achieved in two ways. First, ERK can activate Elk-1 to increase *cfos* transcription. Second, ERK can activate RSK, which translocates to the nucleus and activates *cfos* transcription directly (Godeny and Sayeski, 2006) or indirectly via CREB (Wang and Prywes, 2000). Since cFos was shown to regulate BDNF expression *in vivo* and *in vitro* in neurons (Zhang et al., 2002a) and considering that melanocortins can induce cFos activation in the hypothalamus (McMinn et al., 2000; Singru et al., 2012), we next tested whether MC4R activation induces cFos expression in astrocytes. We found that 1 h treatment with NDP-MSH increases cFos mRNA levels (Fig. 4A) as well as cFos protein (Fig. 4B). Moreover, treatment with NDP-MSH induced cFos translocation to the nucleus of astrocytes (Fig. 4B). RSK inhibitor BRD7389 (1 μM) blocked the increased in cFos expression induced by NDP-MSH in astrocytes whereas the stimulatory effect of NDP-MSH on cFos expression was not observed in the presence of Src inhibitor PP2 (2 μM), indicating that RSK and Src are involved in this effect (Fig. 4A).

3.4. α-MSH induces BDNF expression as well as ERK1/2 and cFos activation in the hypothalamus of male rats

Since we have previously shown that MC4R exerted anti-inflammatory effects in the HMB (Caruso et al., 2004), we next investigated whether BDNF expression is increased by melanocortins in the HMB of male rats. To this end, we injected male rats ip with vehicle (saline) or α-MSH (0.5 mg/kg) and examined BDNF mRNA levels by RT-qPCR in the HMB 3 h after a single injection (**3h**) or 48 h after two daily injections (**48h**). Immunohistochemistry of BDNF showed that BDNF is not only predominantly expressed in neurons but it is also expressed in astrocytes (Fig. 5A). BDNF mRNA levels in the HMB were increased 3 h after α-MSH injection but mRNA returned to basal levels after 48 h treatment (Fig. 5B). BDNF protein expression was also increased by α-MSH at 48 h (Fig. 5D). Moreover, MC4R gene expression was increased in the HMB by α-MSH at 3 and 48 h (Fig. 5C) whereas MC4R protein expression was increased by α-MSH after 48 h (Fig. 5E). Next, we determined MC4R signaling in the HMB and found that levels of pERK were increased by α-MSH treatment after 3 h (Fig. 6A). Although we detected no changes in cFos mRNA after 3 h (data not shown), by 48 h immunohistochemistry shows a remarkable increase in the number of cFos-positive cells in the HMB of rats treated with α-MSH (Fig. 6B and C). cFos-positive cells were predominantly neurons although few cFos-positive cells might correspond to glial cells (Fig. 6B). Thus, melanocortin signaling in HMB involves ERK-cFos activation mainly in neurons but astrocytes may also contribute to this effect.

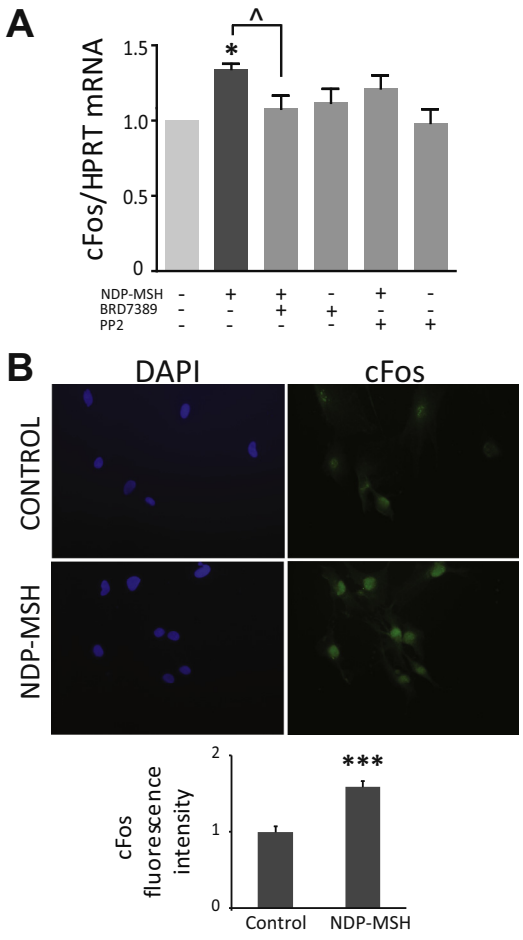

Fig. 4. NDP-MSH increases cFos expression and activation in astrocytes. (A) Astrocytes were preincubated for 15 min with inhibitors of RSK (**BRD7389** 1 μM) or Src (**PP2 2μM**) and then treated with 1 μM **NDP-MSH** for 1 h. Total RNA was extracted and processed for RT-qPCR. Values are the mean of cFos/HPRT ratio compared to control group of 6 independent experiments and were analyzed by one-way ANOVA. $^{*}p < 0.05$ vs. control group and $^{\wedge}p < 0.05$ vs. NDP-MSH. (B) Astrocytes were incubated with serum free medium and after stabilization for 24 h they were treated with 1 μM **NDP-MSH** for 2 h. Cells were fixed and cFos (green) was identified by indirect immunofluorescence staining and the nucleus with DAPI (blue). Semiquantification of cFos fluorescence intensity was determined in 5–7 pictures from 2 independent experiments. Data were analyzed by Student t test. $^{***}p < 0.001$ vs. control group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

A variety of melanocortin effects in the brain involve MC4R activation. MC4R is known to participate in reproductive function, energy homeostasis, inflammation, and neuroprotection (Tao, 2010). Recently, MC4R intervention in nociceptive, cognitive and mood disorders was reviewed (Caruso et al., 2014). This receptor has neuroprotective effects in a model of cerebral ischemia (Giuliani et al., 2006) and we previously demonstrated that melanocortins, through central MC4Rs, can abolish memory impairment induced by interleukin-1β (Gonzalez et al., 2009). Indeed, melanocortins inhibit the decrease in BDNF expression induced by interleukin-1β in rat hippocampus (Gonzalez et al., 2013). MC4R involvement in energy homeostasis was evident by targeted disruption of the *Mc4r* gene that causes the obesity–diabetes syndrome (Huszar et al., 1997). BDNF expression was shown to be induced in the ventromedial hypothalamus by an α-MSH analog (Xu et al., 2003), suggesting that BDNF is an important effector of melanocortin control of energy balance. Our previous results demonstrated that MC4R activation exerts anti-inflammatory effects in the hypothalamus of male rats (Caruso et al., 2004). We also showed that MC4R is the only MCR that is expressed in astrocytes where it exerts anti-inflammatory and anti-apoptotic effects (Caruso et al., 2007) and that its activation increases BDNF expression in these cells (Caruso et al., 2012a).

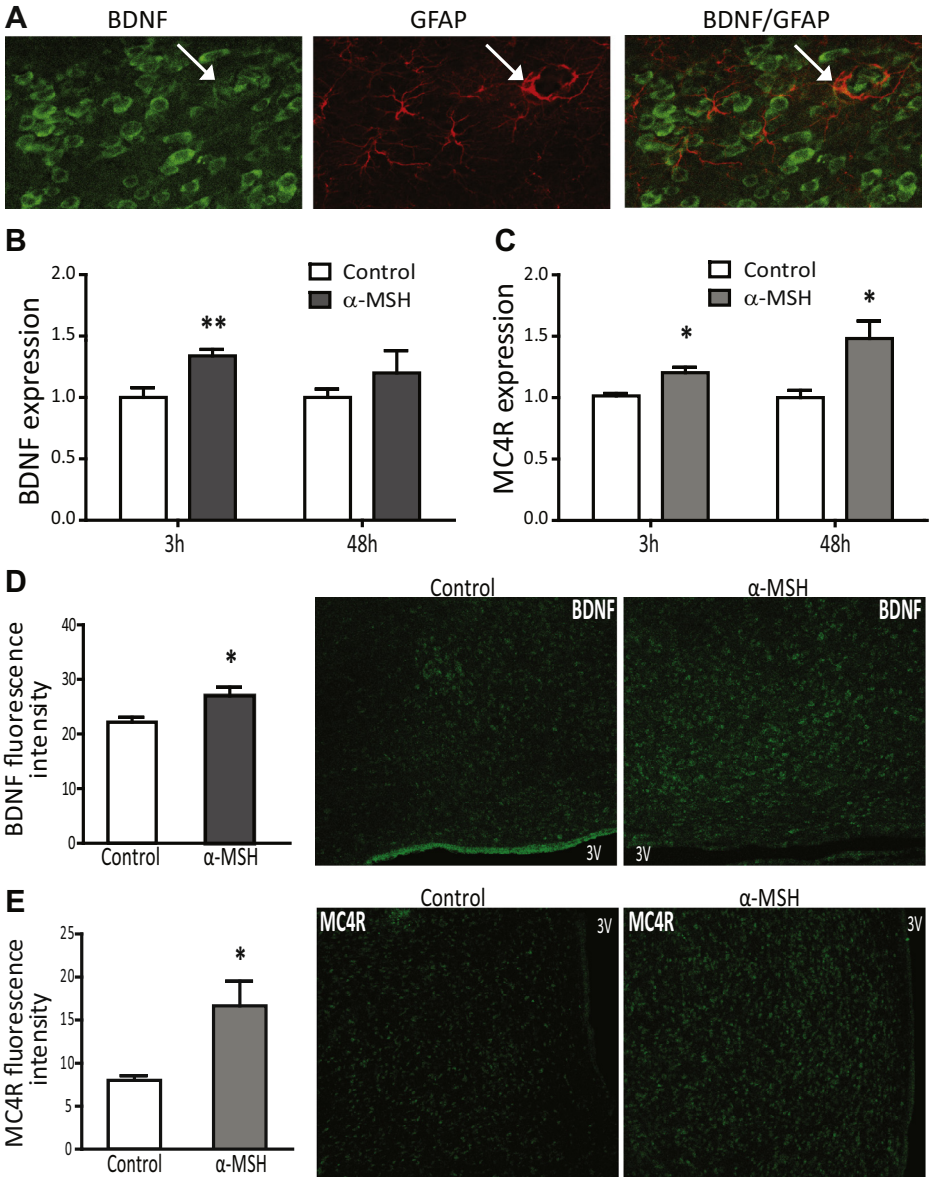

Fig. 5. α-MSH increases BDNF and MC4R expression in rat hypothalamus. (A) Confocal microscopy of HMB immunohistochemistry showing BDNF (green), GFAP (red) and colocalization of BDNF/GFAP staining. (B) Total RNA from HMB of rats injected ip with α-MSH once for 3 h (**3h**) or twice a day for 2 days (**48h**) was extracted and processed for RT-qPCR. Values are the mean of BDNF/HPRT ratio compared to control group of 4–6 animals per group and were analyzed by Student t test. $^{**}p < 0.01$ vs. control group. (C) Total RNA from HMB of rats injected ip with α-MSH once for 3 h (**3h**) or twice a day for 2 days (**48h**) was extracted and processed for RT-qPCR. Values are the mean of MC4R/HPRT ratio compared to control group of 4–6 animals per group and were analyzed by Student t test. $^{*}p < 0.05$ vs. control group. (D) Semiquantification of BDNF immunohistochemistry in HMB of rats treated with α-MSH for **48 h**. Fluorescence intensity for BDNF was determined in four to six HMB sections of 2 animals per group. Data were analyzed by Student t test. $^{*}p < 0.05$ vs. control group. (E) Semiquantification of MC4R immunohistochemistry in HMB of rats treated with α-MSH for **48 h**. Fluorescence intensity for MC4R was determined in four to six HMB sections of 2 animals per group. Data were analyzed by Student t test. $^{*}p < 0.05$ vs. control group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

However, the mechanisms and mediators involved in MC4R action have not been fully elucidated. While we are just beginning to understand MC4R action in astrocytes (Caruso et al., 2013), signaling pathways of MC4R are still poorly known. We have demonstrated that MC4R activation leads to increased BDNF expression through the classic cAMP-PKA-CREB pathway without involving NF-κB in rat astrocytes (Caruso et al., 2012a). This pathway was shown to be important *in vivo* where PKA activity rescued mice from obesity syndrome (Czyzyk et al., 2008). Moreover, CREB activation by melanocortins was shown in neurons of the paraventricular nucleus of the hypothalamus (Sarkar et al., 2002), and in cultured hypothalamic neurons (Caruso et al., 2010), reinforcing the importance of this pathway.

MC4R alternative signaling depends on the cell type where it is expressed (Breit et al., 2011). Nevertheless, ERK activation was shown for all MCRs. MC4R can activate ERK *in vitro* (Chai et al., 2006; Damm et al., 2012; Vongs et al., 2004) and *in vivo* (Daniels et al., 2003; Sutton et al., 2005). Concordantly, our present results show that MC4R agonist NDP-MSH activates ERK1/2 in astrocytes. Moreover, our data show that ERK-RSK pathway is involved in MC4R signaling since both ERK and RSK inhibitors blocked the stimulatory effect of NDP-MSH on BDNF expression. RSK, reported to be an important effector of ERK in transcription regulation (Romeo et al., 2012), is expressed by astrocytes where it participates in chemokine action in these cells (Zhang et al., 2002b). p38 activation in the hippocampus of an Alzheimer animal model was shown to be decreased by

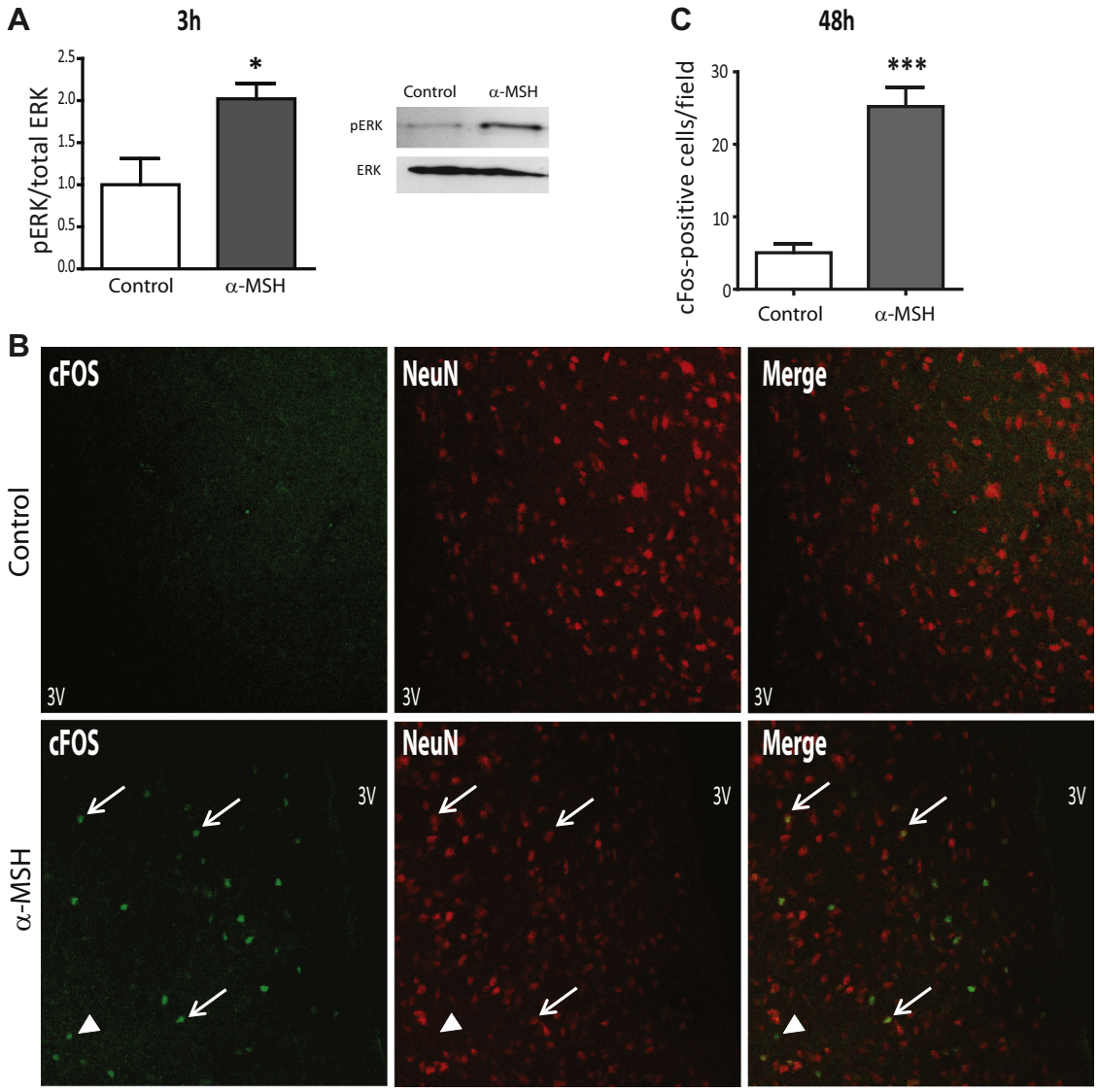

Fig. 6. α-MSH induces ERK and cFos activation in rat hypothalamus. (A) Total protein extract of HMB was subjected to Western blot. Membranes were probed with antibodies against pERK and total ERK. Values represent the pERK/total ERK ratio compared to control group of 3 animals per group and were analyzed by Student t test. $^{*}p < 0.05$ vs. control group. (B) cFos immunohistochemistry. Coronal sections of rat brain were treated with anti-cFos (green) and anti-NeuN (red) antibodies and the positive nuclei were determined in the hypothalamus after 48 h treatment with α-MSH. Arrows show cFos-positive neurons. Arrow head shows cFos-positive cell that is not positive for NeuN staining. (C) Quantification of cFos immunoreactivity was performed in HMB by counting cFos-positive cells in 4 sections from each animal. Values represent the mean number of cFos-positive cells of 2 rats per group and were analyzed by Student t test. $^{***}p < 0.001$ vs. control group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

an MC4R agonist (Giuliani et al., 2014) and NDP-MSH inhibited both basal and insulin-induced JNK activity in HEK293 cells expressing MC4R (Chai et al., 2009). However, our present results show that p38 and JNK do not participate in MC4R-induced BDNF expression in astrocytes. Moreover, we show that JNK inhibition by SP600125 induces an increase in BDNF expression in these cells. Since inhibition of JNK by SP600125 was reported to induce CREB activation (Vaishnav et al., 2003), it is possible that this mechanism may be responsible for the increased BDNF expression found after SP600125 treatment in astrocytes. Consequently, NDP-MSH and SP600125 induce an additive increase in BDNF expression.

In this work we also demonstrated that ERK activation by MC4R involves cAMP in astrocytes since ERK phosphorylation induced by NDP-MSH is decreased by an AC inhibitor. ERK activation was also demonstrated for other MCRs such as MC1R, where it was shown to be cAMP-independent and not induced by forskolin (Herraiz et al., 2011), and also for MC5R, where ERK activation is cAMP and PKA independent (Rodrigues et al., 2009). We previously showed that PKA inhibition partially prevented the increase in BDNF expression induced by NDP-MSH (Caruso et al., 2012a), suggesting that other signaling pathways may be involved in this effect. Now, we show that ERK activation by MC4R does not involve PKA which is in agreement with previous reports (Chai et al., 2006; Vongs et al., 2004). These findings indicate that effects over the ERK pathway in astrocytes are cAMP-dependent but PKA-independent.

We also show here that ERK activation by NDP-MSH was completely abolished by inhibition of PI3K. In fact, a PI3K inhibitor was reported to block ERK activation induced by MC4R in stably transfected CHO-K1 cells (Vongs et al., 2004). The same effect was also shown for MC5R which induces ERK activation in a PI3K-dependent but Akt-independent way (Rodrigues et al., 2009). Our data also show that inhibition of PI3K completely blocked BDNF expression induced by NDP-MSH. In the classic PI3K/Akt pathway PI3K activates PDK1 which in turn activates Akt. However, PDK1 or Akt inhibition did

not modify BDNF expression and MC4R activation did not induce Akt phosphorylation, indicating that PDK1 and Akt are not involved in MC4R signaling. Indeed, ERK activation through PI3K without involving Akt was reported for other GPCRs as well (Montiel et al., 2006).

One of the mechanisms of ERK activation by GPCRs is the Src family of non receptor tyrosine kinases. The binding of arrestins to GPCR can recruit Src that can activate Ras/Raf GTPases which then activate the MAPK cascade leading to ERK activation (Luttrell et al., 1999). Alternative Gs proteins can directly activate Src resulting in ERK activation (Ma et al., 2000). In astrocytes, our results show that Src inhibition completely abolished BDNF expression induced by MC4R activation. Moreover, Src inhibition decreased basal and NDP-MSH-induced ERK activation. These results suggest that Src helps maintain basal ERK activation in these cells and that it is also involved in MC4R signaling. Other GPCRs were also shown to induce ERK activation through PI3K and Src (Montiel et al., 2006). MC1R signaling also involves Src-mediated transactivation of the stem cell factor receptor (Herraiz et al., 2011). Whether or not MC4R activation can induce transactivation of tyrosine kinase receptors remains to be tested.

cFos is a transcription factor that modulates the physiological response to growth factors and to stressful stimuli in cells and therefore it is essential in regulating cell survival. Activation of ERK can induce *cfos* transcription via CREB or RSK (Wang and Prywes, 2000). Moreover, cFos can regulate BDNF expression in neurons (Zhang et al., 2002a). Our results demonstrate that MC4R activation induces cFos expression and activation in astrocytes. Concordantly, it was recently reported that cFos is induced through MC5R activation (Rodrigues et al., 2012). cFos expression induced by MC4R activation involves ERK-RSK pathway since our data show that it can be blocked by RSK inhibition. Also, our results show that Src is involved in the increased cFos expression induced by MC4R activation, in line with its involvement on ERK activation through MC4R and further implying that Src mediates MC4R action in astrocytes. Thus, MC4R signaling in astrocytes activates ERK-RSK-cFos pathway in a Src/PI3K-dependent manner.

In order to investigate whether MC4R-induced effects on astrocytes observed here were also taking place in the brain as a general mechanism, we performed *in vivo* experiments. We found that ip administration of α-MSH increased BDNF expression in rat HMB, indicating that melanocortins exert an effect on the HMB similar to that exerted by MC4R activation on astrocytes. Concordantly, central administration of MTII (a MCR agonist) induces BDNF expression in the ventromedial hypothalamus (Xu et al., 2003). Also, BDNF was reported to be a mediator of the control of food intake exerted by MC4R activation in the dorsal vagal complex (Bariohay et al., 2009). Now, we show that systemic administration of α-MSH induced ERK and cFos activation in the HMB. Congruently, MC4R activation in the solitary nucleus was shown to involve ERK since this MAPK mediates melanocortin-induced food intake reduction in rats (Sutton et al., 2005) and central administration of α-MSH induces increased-cFos expression in the paraventricular nucleus (McMinn et al., 2000). These results suggest that the effect of α-MSH on BDNF and MC4R expression in the HMB is due mainly to activation of neuronal MC4R which are more abundantly expressed in these cells. Our data also show that cFos was activated predominantly in hypothalamic neurons by α-MSH. Therefore, effects of melanocortins on the HMB involve mainly neuronal cells. Nevertheless, we propose that astrocytes may contribute in a lesser extent to melanocortin action in the HMB by releasing BDNF through MC4R activation as well. BDNF increase by melanocortins in the HMB may contribute to melanocortin neuroprotective effects as well as to the control of energy balance within the brain.

MC3R is also expressed in the hypothalamus where it is involved in energy homeostasis (Chen et al., 2000). MC3R agonist was shown to induce ERK activation in the dorsomedial hypothalamus and cFos activation induced by restricted feeding in the dorsomedial region of the hypothalamus was impaired in MC3R knock-out mice (Begriche et al., 2012). However, our data show that ip administration of α-MSH activated cFos in the HMB including paraventricular, dorsomedial and ventromedial hypothalamus and indicate that α-MSH treatment increased MC4R expression in the HMB. Although we cannot exclude MC3R involvement in these effects on the HMB, it is likely that MC4R may have a role in melanocortin effects on the HMB. Since α-MSH induces the same effects in the HMB as MC4R activation in astrocytes and increases MC4R expression in the HMB, MC4R may play a predominant role in melanocortin action in the HMB, astrocyte MC4R contributing to these effects in the brain.

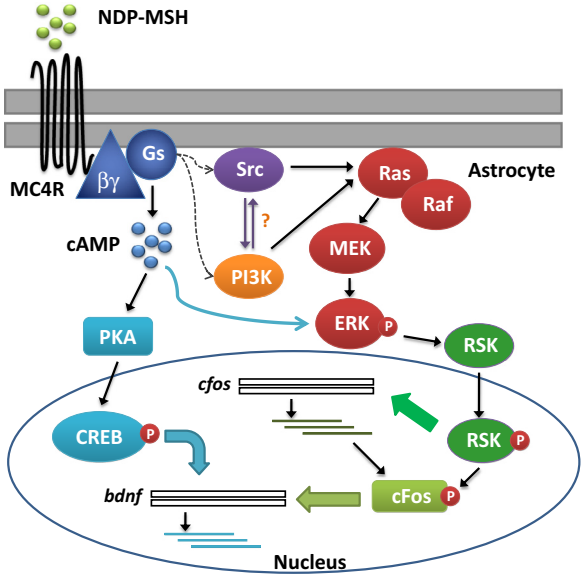

Fig. 7. MC4R signaling in astrocytes MC4R activation by NDP-MSH activates Gs protein leading to cAMP production with the consequent activation of PKA and CREB resulting in increased transcription of *bdnf* gene. Gs may recruit Src and/or PI3K. Src can directly or indirectly activate PI3K. PI3K may also activate Src. Both kinases lead to ERK activation, through MEK and Ras/Raf GTPases. ERK activation can also be induced by cAMP directly or indirectly in a PKA-independent fashion. ERK activates RSK which in turn activates and increases transcription of cFos. Finally, cFos increases transcription of *bdnf* gene in astrocytes.

Therefore, our results provide new insights into mechanisms of MC4R action. We have already shown that MC4R activation by NDP-MSH induced cAMP production with subsequent PKA and CREB activation leading to increased *bdnf* transcription (Fig. 7). We hypothesize that Gs activation by MC4R recruits Src or PI3K. Src directly or indirectly activates PI3K and/or PI3K activates Src. Here, we show that MC4R signaling activates ERK-RSK-cFos pathway, through Src and PI3K, leading to increased transcription of *bdnf* gene in astrocytes (Fig. 7). Astrocytes have important functions in maintaining brain homeostasis and are influenced by MC4R action. The present results add valuable knowledge concerning MC4R signaling and suggest that BDNF may be an important effector of MC4R-induced effects in the brain. They also contribute to the better understanding of the physiological effects of melanocortins within the brain.

Acknowledgements

This work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT), the University of Buenos Aires (UBA) and the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

References

Bariohay, B., et al., 2009. Brain-derived neurotrophic factor/tropomyosin-related kinase receptor type B signaling is a downstream effector of the brainstem melanocortin system in food intake control. Endocrinology 150, 2646–2653.

Begriche, K., et al., 2012. Melanocortin-3 receptors are involved in adaptation to restricted feeding. Genes Brain Behav. 11, 291–302.

Breit, A., et al., 2011. Alternative G protein coupling and biased agonism: new insights into melanocortin-4 receptor signalling. Mol. Cell. Endocrinol. 331, 232–240.

Caruso, C., et al., 2004. Alpha-melanocyte-stimulating hormone through melanocortin-4 receptor inhibits nitric oxide synthase and cyclooxygenase expression in the hypothalamus of male rats. Neuroendocrinology 79, 278–286.

Caruso, C., et al., 2007. Activation of melanocortin 4 receptors reduces the inflammatory response and prevents apoptosis induced by lipopolysaccharide and interferon-gamma in astrocytes. Endocrinology 148, 4918–4926.

Caruso, C., et al., 2010. Alpha-melanocyte-stimulating hormone modulates lipopolysaccharide plus interferon-gamma-induced tumor necrosis factor-alpha expression but not tumor necrosis factor-alpha receptor expression in cultured hypothalamic neurons. J. Neuroimmunol. 227, 52–59.

Caruso, C., et al., 2012a. Melanocortin 4 receptor activation induces brain-derived neurotrophic factor expression in rat astrocytes through cyclic AMP-protein kinase A pathway. Mol. Cell. Endocrinol. 348, 47–54.

Caruso, C., et al., 2012b. Melanocortins: anti-inflammatory and neuroprotective peptides. In: Loh, L.M.M.A.S. (Ed.), Neurodegeneration. Intech, Croatia, pp. 93–120.

Caruso, C., et al., 2013. Astrocytes: new targets of melanocortin 4 receptor actions. J. Mol. Endocrinol. 51, R33–R50.

Caruso, V., et al., 2014. Synaptic changes induced by melanocortin signalling. Nat. Rev. Neurosci. 15, 98–110.

Catania, A., 2008. Neuroprotective actions of melanocortins: a therapeutic opportunity. Trends Neurosci. 31, 353–360.

Chai, B., et al., 2006. Melanocortin-4 receptor-mediated inhibition of apoptosis in immortalized hypothalamic neurons via mitogen-activated protein kinase. Peptides 27, 2846–2857.

Chai, B., et al., 2009. Melanocortin-4 receptor activation inhibits c-Jun N-terminal kinase activity and promotes insulin signaling. Peptides 30, 1098–1104.

Chen, A.S., et al., 2000. Inactivation of the mouse melanocortin-3 receptor results in increased fat mass and reduced lean body mass. Nat. Genet. 26, 97–102.

Chu, H., et al., 2012. Melanocortin 4 receptor induces hyperalgesia and allodynia after chronic constriction injury by activation of p38 MAPK in DRG. Int. J. Neurosci. 122, 74–81.

Czyzyk, T.A., et al., 2008. Disruption of the RIIbeta subunit of PKA reverses the obesity syndrome of Agouti lethal yellow mice. Proc. Natl. Acad. Sci. U.S.A. 105, 276–281.

Damm, E., et al., 2012. Melanocortin-induced PKA activation inhibits AMPK activity via ERK-1/2 and LKB-1 in hypothalamic GT1-7 cells. Mol. Endocrinol. 26, 643–654.

Daniels, D., et al., 2003. Melanocortin receptor signaling through mitogen-activated protein kinase in vitro and in rat hypothalamus. Brain Res. 986, 1–11.

Durand, D., et al., 2011. Reduced cAMP, Akt activation and p65-c-Rel dimerization: mechanisms involved in the protective effects of mGluR3 agonists in cultured astrocytes. PLoS ONE 6, e22235.

Fomina-Yadlin, D., et al., 2010. Small-molecule inducers of insulin expression in pancreatic alpha-cells. Proc. Natl. Acad. Sci. U.S.A. 107, 15099–15104.

Giuliani, D., et al., 2006. Both early and delayed treatment with melanocortin 4 receptor-stimulating melanocortins produces neuroprotection in cerebral ischemia. Endocrinology 147, 1126–1135.

Giuliani, D., et al., 2014. Melanocortins protect against progression of Alzheimer's disease in triple-transgenic mice by targeting multiple pathophysiological pathways. Neurobiol. Aging 35, 537–547.

Godeny, M.D., Sayeski, P.P., 2006. ERK1/2 regulates ANG II-dependent cell proliferation via cytoplasmic activation of RSK2 and nuclear activation of elk1. Am. J. Physiol. Cell Physiol. 291, C1308–C1317.

Gonzalez, P., et al., 2013. Molecular mechanisms involved in interleukin 1-beta (IL-1beta)-induced memory impairment. Modulation by alpha-melanocyte-stimulating hormone (alpha-MSH). Brain Behav. Immun. 34, 141–150.

Gonzalez, P.V., et al., 2009. Memory impairment induced by IL-1beta is reversed by alpha-MSH through central melanocortin-4 receptors. Brain Behav. Immun. 23, 817–822.

Herraiz, C., et al., 2011. Signaling from the human melanocortin 1 receptor to ERK1 and ERK2 mitogen-activated protein kinases involves transactivation of cKIT. Mol. Endocrinol. 25, 138–156.

Huszar, D., et al., 1997. Targeted disruption of the melanocortin-4 receptor results in obesity in mice. Cell 88, 131–141.

Lewin, G.R., Barde, Y.A., 1996. Physiology of the neurotrophins. Annu. Rev. Neurosci. 19, 289–317.

Liebmann, C., 2001. Regulation of MAP kinase activity by peptide receptor signalling pathway: paradigms of multiplicity. Cell. Signal. 13, 777–785.

Luttrell, L.M., et al., 1999. Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. Science 283, 655–661.

Ma, Y.C., et al., 2000. Src tyrosine kinase is a novel direct effector of G proteins. Cell 102, 635–646.

Machado, I., et al., 2010. Alpha-melanocyte-stimulating hormone (alpha-MSH) reverses impairment of memory reconsolidation induced by interleukin-1 beta (IL-1 beta) hippocampal infusions. Peptides 31, 2141–2144.

Marsh, D.J., et al., 1999. Response of melanocortin-4 receptor-deficient mice to anorectic and orexigenic peptides. Nat. Genet. 21, 119–122.

McMinn, J.E., et al., 2000. Effect of intracerebroventricular alpha-MSH on food intake, adiposity, c-Fos induction, and neuropeptide expression. Am. J. Physiol. Regul. Integr. Comp. Physiol. 279, R695–R703.

Montiel, M., de la Blanca, E.P., Jimenez, E., 2006. P2Y receptors activate MAPK/ERK through a pathway involving PI3K/PDK1/PKC-zeta in human vein endothelial cells. Cell. Physiol. Biochem. 18, 123–134.

Najafov, A., et al., 2011. Characterization of GSK2334470, a novel and highly specific inhibitor of PDK1. Biochem. J. 433, 357–369.

Paxinos, G., Watson, C., 1997. The Rat Brain in Stereostaxic Coordinates. Academic Press, United States of America.

Perez-Cadahia, B., Drobic, B., Davie, J.R., 2011. Activation and function of immediate-early genes in the nervous system. Biochem. Cell Biol. 89, 61–73.

Rodrigues, A.R., et al., 2009. Melanocortin 5 receptor activates ERK1/2 through a PI3K-regulated signaling mechanism. Mol. Cell. Endocrinol. 303, 74–81.

Rodrigues, A.R., Almeida, H., Gouveia, A.M., 2012. Melanocortin 5 receptor signaling and internalization: role of MAPK/ERK pathway and beta-arrestins 1/2. Mol. Cell. Endocrinol. 361, 69–79.

Romeo, Y., Zhang, X., Roux, P.P., 2012. Regulation and function of the RSK family of protein kinases. Biochem. J. 441, 553–569.

Sarkar, S., Legradi, G., Lechan, R.M., 2002. Intracerebroventricular administration of alpha-melanocyte stimulating hormone increases phosphorylation of CREB in TRH- and CRH-producing neurons of the hypothalamic paraventricular nucleus. Brain Res. 945, 50–59.

Selkirk, J.V., et al., 2007. Identification of differential melanocortin 4 receptor agonist profiles at natively expressed receptors in rat cortical astrocytes and recombinantly expressed receptors in human embryonic kidney cells. Neuropharmacology 52, 459–466.

Singru, P.S., et al., 2012. Refeeding-activated glutamatergic neurons in the hypothalamic paraventricular nucleus (PVN) mediate effects of melanocortin signaling in the nucleus tractus solitarius (NTS). Endocrinology 153, 3804–3814.

Sinha, P.S., Schioth, H.B., Tatro, J.B., 2004. Roles of the melanocortin-4 receptor in antipyretic and hyperthermic actions of centrally administered alpha-MSH. Brain Res. 1001, 150–158.

Sutton, G.M., et al., 2005. Melanocortinergic modulation of cholecystokinin-induced suppression of feeding through extracellular signal-regulated kinase signaling in rat solitary nucleus. Endocrinology 146, 3739–3747.

Tao, Y.X., 2010. The melanocortin-4 receptor: physiology, pharmacology, and pathophysiology. Endocr. Rev. 31, 506–543.

Vaishnav, D., et al., 2003. SP600125, an inhibitor of c-jun N-terminal kinase, activates CREB by a p38 MAPK-mediated pathway. Biochem. Biophys. Res. Commun. 307, 855–860.

Vongs, A., Lynn, N.M., Rosenblum, C.I., 2004. Activation of MAP kinase by MC4-R through PI3 kinase. Regul. Pept. 120, 113–118.

Wang, Y., Prywes, R., 2000. Activation of the c-fos enhancer by the erk MAP kinase pathway through two sequence elements: the c-fos AP-1 and p62TCF sites. Oncogene 19, 1379–1385.

Xu, B., et al., 2003. Brain-derived neurotrophic factor regulates energy balance downstream of melanocortin-4 receptor. Nat. Neurosci. 6, 736–742.

Yeatman, T.J., 2004. A renaissance for SRC. Nat. Rev. Cancer 4, 470–480.

Zhang, J., et al., 2002a. c-fos regulates neuronal excitability and survival. Nat. Genet. 30, 416–420.

Zhang, Y., et al., 2002b. RANTES-mediated chemokine transcription in astrocytes involves activation and translocation of p90 ribosomal S6 protein kinase (RSK). J. Biol. Chem. 277, 19042–19048.

Zhu, J., et al., 2004. From the cyclooxygenase-2 inhibitor celecoxib to a novel class of 3-phosphoinositide-dependent protein kinase-1 inhibitors. Cancer Res. 64, 4309–4318.