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Oroxylin A increases BDNF production by activation of MAPK–CREB pathway in rat primary cortical neuronal culture

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ABSTRACT

Oroxylin A (5,7-dihydroxy-6-methoxyfavone) is a flavonoid compound originated from the root of *Scutellaria baicalensis* Georgi. Our previous reports suggested that oroxylin A improves memory function in rat, at least in part, by its antagonistic effects on $GABA_A$ receptor. In addition, oroxylin A protects neurons from ischemic damage by mechanisms currently not clear. In this study we determined whether oroxylin A modulates the level of brain derived neurotrophic factor (BDNF) in primary rat cortical neuronal culture, which is well known for its role on neuronal survival, neurogenesis, differentiation of neurons and synapses and learning and memory. Treatment of oroxylin A for 3–48 h increased BDNF expression which was analyzed by ELISA assay and Western blot analysis. Oroxylin A induced slow but sustained increases in intracellular calcium level and activated ERK1/2 mitogen activated protein kinase (MAPK). In addition, oroxylin A phosphorylated cyclic AMP response element binding protein (CREB) at Ser 133 in concentration and time dependent manner. Pretreatment with the MAPK inhibitor PD98059 (10 μM) attenuated phosphorylation of ERK1/2 and CREB as well as BDNF production, which suggests that oroxylin A regulates BDNF production by activating MAPK–CREB pathway. $GABA_A$ antagonist bicuculline mimicked the effects of oroxylin A on BDNF production as well as MAPK–CREB pathway. Increase in intracellular Ca^{2+} concentration, phosphorylation of ERK1/2 and CREB, and BDNF expression by oroxylin A was blocked by NMDA receptor inhibitor MK-801 (10 μM) as well as tetrodotoxin (TTX, 0.5 and 1 μM). The results from the present study suggest that the calcium and p-CREB dependent induction of BDNF expression, possibly via activation of synaptic NMDA receptor through the blockade of $GABA_A$ activity in cortical neuronal circuitry, might be responsible for the neuroprotective or memory enhancing effects of oroxylin A.

1. Introduction

Oroxylin A (5,7-dihidrixy-6-methoxyflavone) is a flavonoid compound originated from the root of *Scutellaria baicalensis* Georgi. Oroxylin A inhibits lipopolysaccharide (LPS) induced inducible nitric oxide (iNOS) and cyclooxgenase2 (COX-2) gene expression via suppression of nuclear factor-kappa B (NF-κB) activation (Chen et al., 2000) and suppresses superoxide and nitric oxide generation (Jiwajinda et al., 2002), suggesting that oroxylin A has an anti-inflammatory and anti-oxidative activity. In addition, we have reported that oroxylin A ameliorates memory dysfunction induced by transient bilateral common carotid artery occlusion (2VO) (Kim et al., 2006), scopolamine (Kim et al., 2007), and Aβ (25–35) (Kim et al., 2008). We reported that oroxylin A increased phosphorylation of cAMP response element-binding protein (CREB) and brain derived neurotrophic factor (BDNF) positive neuronal cell numbers in hippocampal CA1 and dentate gyrus regions of 2VO mice (Kim et al., 2006). We have concluded that memory enhancing effects of (sub)chronic administration of oroxylin A is mediated by neuroprotective effects including increased production of neurotrophic factors such as BDNF and inhibition of microglial activation (Kim et al., 2006, 2008), while short-term improvement against amnesic effects of scopolamine is mediated by $GABA_A$-antagonistic property of oroxylin A (Huen et al., 2003b; Kim et al., 2007). However,

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the mechanism by which oroxylin A regulates BDNF expression is unclear, yet.

BDNF is a member of the neurotrophin family (Lewin, 1996). BDNF plays important roles in CNS such as protection of neuronal degeneration (Lindholm et al., 1993), differentiation of hippocampal and cortical neurons (Ip et al., 1993; Croll et al., 1994; Nawa et al., 1994; Marty et al., 1996) and regulation of long term memory (Bekinschtein et al., 2008).

One of the most well known and immediate transcriptional regulator of BDNF gene expression is CREB (Tao et al., 1998). CREB is a transcription factor that regulates genes associated with neuronal survival, memory consolidation and synaptic plasticity (Impey et al., 2004). CREB is phosphorylated and become transcriptionally active in response to the stimuli of growth factors, hormones and neuronal activities (Shaywitz and Greenberg, 1999). Phosphorylation of CREB on serine 133 recruits p300/CBP and activates gene transcription (Kwok et al., 1994).

In this study, we investigated the mechanism by which oroxylin A regulates BDNF expression in rat primary cortical neuronal cultures.

2. Materials and methods

2.1. Materials

Neurobasal medium (NBM), Dulbeco's modified Eagle's medium/F12 (DMEM/F12), fetal bovine serum (FBS) and Hanks' balanced salt solution (HBSS) were obtained from Gibco BRL (Grand Island, NY). B-27 supplement was purchased from Invitrogen (Carlsbad, CA). Rabbit polyclonal anti phopho-CREB was purchased from Upstate (Lake Placid, NY). Rabbit monoclonal anti CREB and mouse monoclonal anti phopho-ERK1/2 antibodies were from Cell Signaling Technology (Beverly, MA, USA). Anti ERK2 and anti BDNF (sc-546, mature form: 14 kDa) were from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Fluo-3, AM ester and Pluronic F-127 were purchased from Invitrogen (Carlsbad, CA). PD98059 was purchased from Calbiochem (La Jolla, CA) and bicuculline was from Tocris (Bristol, UK). Dizocilpine (MK-801) and poly-D-lysine (PDL) were from Sigma–Aldrich Co (St. Louis, MO). Oroxylin A was obtained from Korean Food and Drug Administration (KFDA, Seoul). Tetrodotoxin (TTX) and muscimol were purchased from Tocris (Bristol, UK).

2.2. Cell cultures

Primary cortical neurons were isolated from cerebral cortex of embryonic day 16 (E16) Sprague–Dawley rat as previously described (Wang et al., 1998). Dissociated cerebral cortex was digested in calcium and magnesium free Hanks' balanced salt solution (HBSS) and re-suspended in Neurobasal medium (NBM) supplemented with 2% B-27. Cells were plated on PDL pre-coated multiwell plates or 35 mm plating dishes. The cultures were kept in a humidified atmosphere (10% CO_2) at 37 °C. Experiments were performed to use 10–12 days old cultures.

Hippocapal neurons were prepared according to Brewer's methods with the slight modification (Brewer et al., 1993). In brief, hippocampus was dissected from E18 SD rat, dissociated mechanically, and digested with trypsin. Cultures were plated on PDL precoated multiwell plates and maintained in serum free NBM containing 2% B-27. The cultures were grown in a 10% CO_2 incubator for 12 days.

Astrocytes were cultured as indicated earlier (Park et al., 2009). Briefly 1–3 days old SD rats were decapitated. After removal of the meninges, the cortex was passed through a 100-μm mesh filter in DMEM/F12 containing 10% FBS. The cells were plated on PDL pre-coated 75 cm^2 flask. After 7 days, cells were sub-cultured on PDL precoated multiwall plates and kept 3–4 days to adapt. Cells were maintained in a humidified atmosphere (5% CO_2) at 37 °C and exchanged media every third day.

2.3. Calcium imaging

Cortical neurons were loaded with 10 μM Fluo-3 AM and 0.02% of Pluronic F-127 in normal medium and incubated for 30 min at 37 °C. Cortical neurons were washed out with HBSS and then incubated for another 30 min complete de-esterification of intracellular AM esters. Fluorescence was imaged by using confocal microscope (TCS-SP, Leica, Heidelberg, Germany) at 15 s interval for 15 min after oroxylin A treatment. Excitation and emission wavelengths were 506 nm and 526 nm, respectively. Fluorescence changes in the cell bodies of individual cells were analyzed using Image J programs. Data were expressed as $\Delta F/F_0$ manner (Y axis).

2.4. BDNF E_{max} immunoassay system

The concentration of BDNF secreted in the culture medium of neuron was determined by using the E_{max} Immunoassay system (Promega, Madison, WI). BDNF enzyme-linked immunosorbent assay (ELISA) was performed as suggested by the manufacturer. In brief, 96 well plates were coated with anti-BDNF mAb diluted with carbonate coating buffer (pH 9.7) at 4 °C overnight. The plates were blocked by block and sample 1× buffer for 1 h at room temperature. The plates were incubated with BDNF standards or test samples for 2 h at room temperature. And then, plates were incubated 2 h with anti-human BDNF pAb followed by anti-IgY HRP conjugate for 1 h. TMB One Solution was used to develop color and 1 N hydrochloric acid was used to stop the reaction. The absorbance was measured at 450 nm with an ELISA reader (Tecan Trading AG, Switzerland).

2.5. Western blotting analysis

Treated cells were washed with PBS and lysed with 2× sample buffer. The samples were separated by 10% SDS-PAGE gels. After electrophoresis, proteins were electrically transferred to nitrocellulose (NC) membrane and blocked with 1 μg/ml polyvinyl alcohol for 30 min at RT. The membranes were incubated overnight at 4 °C with the appropriate primary antibodies (anti-BDNF 1:2500, anti-phopho-ERK 1:2500, anti-phospho-CREB 1:5000, anti-ERK 1:5000, anti-CREB 1:5000). The anti-BDNF antibody used in this study specifically recognizes 14 kDa mature BDNF. Membranes were washed three times with Tris-buffered saline containing 0.1% Tween20 (TBS–T) and then incubated with peroxidase conjugated secondary antibody for 2 h at RT. Blots were washed again with TBS–T for three times and detected by enhanced chemiluminescence method (Amersham, Buckinghamshire, UK).

2.6. Real time RT-PCR

Total RNA was extracted from neurons using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and 2 μg of total RNA was converted to cDNA Maxime RT PreMix Kit (iNtRON Biotechnology, Seoul) according to the manufacturer's protocol. The cDNA was amplified with exon IV BDNF specific forward and reverse primers. The real time RT-PCR was performed using a Light Cycler (Roche applied Science, Indianapolis, IN, USA) with SYBR Green mix (Fermentas, Glen Burnie, Maryland). The real-time PCR was performed at 95 °C for 1 s, 56 °C for 5 s, and 72 °C for 10 s, with a maximum of 40 cycles. The following primers were used for BDNF (accession number EF125679) as reported by Yasuda et al. (2009) as well as for glyceraldehyde 3-phosphate dehydrogenase (GAPDH, accession number M17701).

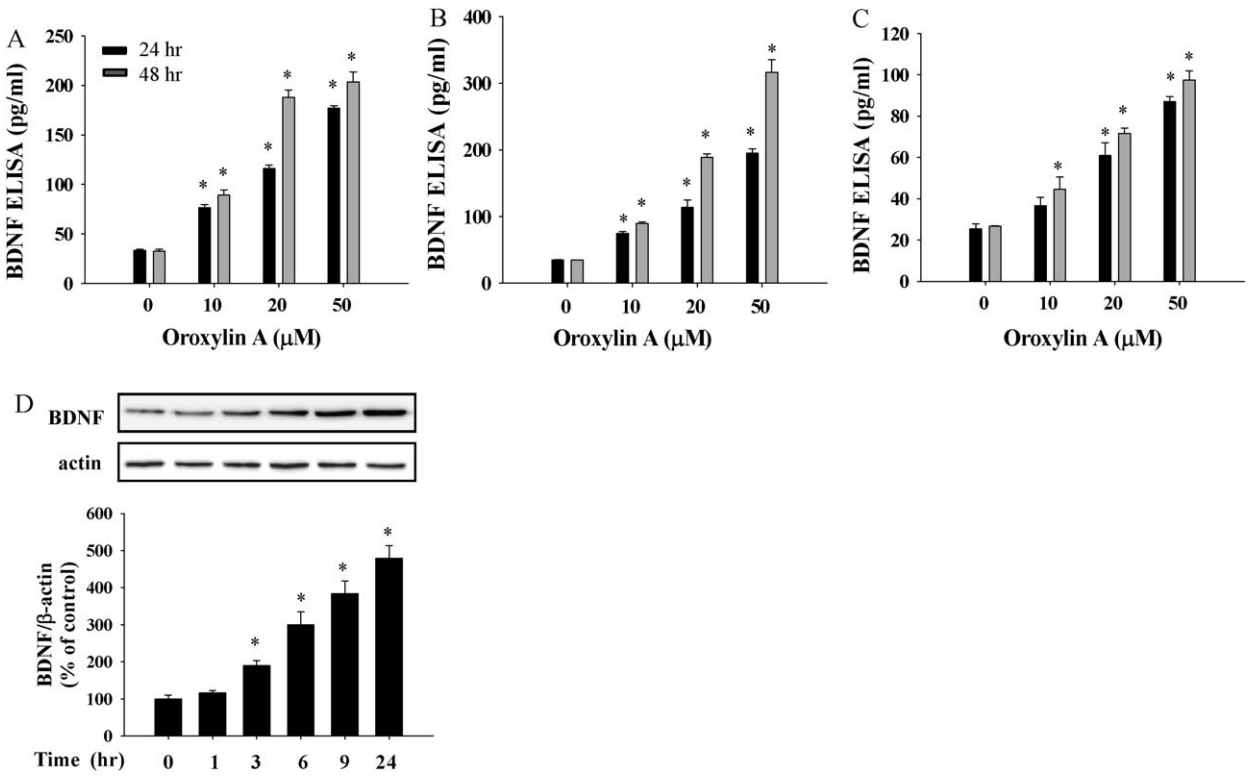

Fig. 1. Oroxylin A increased BDNF release in rat primary cortical neuronal culture. (A) Primary cortical neuronal culture were treated with oroxylin A for 24 or 48 h and BDNF level in the culture supernatant was analyzed by BDNF E_{max} immunoassay system. Oroxylin A increased BDNF level in a concentration dependant manner. Similar results were obtained with rat primary hippocampal neuron (B) and astrocytes (C). (D) Oroxylin A (20 μM) was treated for various time points (1–24 h) in cortical neuronal culture and the expression level of mature form of BDNF was analyzed by Western blot. $^*p < 0.01$ versus untreated control values ($n = 3$).

For BDNF,

exon IV forward primer: 5′-GCT GCC TTG ATG TTT ACT TTG-3′,
exon IV reverse primer: 5′-ATG GGA TTA CAC TTG GTC TCG T-3′

For GAPDH,

forward primer: 5′-TCC CTC AAG ATT GTC AGC AA-3′,
reverse primer: 5′-AGA TCC ACA ACG GAT ACA TT-3′

After all the reactions were finished, data were compiled automatically by the equipment (Roche, Indianapolis, IN, USA).

2.7. Statistics

Data are analyzed by one way analysis of variance (ANOVA) followed by Newman–Keuls test as a *post hoc* test. p value less than 0.05 was considered statistically significant. Results are expressed as the mean ± standard error of mean (SEM).

3. Results

To investigate whether oroxylin A induces BDNF expression, we determined BDNF release in culture supernatants of rat primary neuronal culture by immunoassay. Oroxylin A was treated on cultured rat primary cortical neuron for 24 and 48 h in various concentrations. BDNF level was increased by oroxylin A in a concentration-dependent manner, 400% of control level at an oroxylin A concentration of 50 μM (Fig. 1A). Similar results were obtained with cultured rat primary hippocampal neuron as well as rat primary astrocytes (Fig. 1B and C). The extent of increase in BDNF release was greater in neurons compared with astrocytes. We also measured the BDNF expression (mature form) by Western blot after 1–24 h incubation with oroxylin A. As expected, a significant increase in mature BDNF protein level was detected after 3 h of oroxylin A treatment (1.90 ± 13.49-fold compared with control) and the increase was maintained up to 24 h (4.79 ± 34.76-fold versus control) (Fig. 1D).

To examine the intracellular signaling pathway with which oroxylin A increases BDNF, the level of phosphorylated ERK (p-ERK) and CREB (p-CREB) was examined by Western blot. Oroxylin A induced phosphorylation of ERK and CREB in a time- (Fig. 2A) and concentration- (Fig. 2B) dependent manner. The phosphorylation of ERK1/2 and CREB was persisted for 1–6 h after oroxylin A treatment. Because CREB phosphorylation is a well known immediate regulator of BDNF expression, these data suggest that oroxylin A induces BDNF expression by regulating CREB phosphorylation.

Since oroxylin A has been suggested to have a $GABA_A$ antagonistic properties (Huen et al., 2003a), we tested whether a $GABA_A$ antagonist, bicuculline increase BDNF level. Similar to the results with oroxylin A, treatment of bicuculline increased BDNF gene expression (Fig. 3A) as well as protein expression (Fig. 3B), which was determined by real-time PCR and Western blot, respectively. Although originally suggested that BDNF gene consist of four 5′ exons (exon I–IV) and a common 3′ exon (exon V) (Metsis et al., 1993; Timmusk and Metsis, 1994), it is now widely accepted that the mouse and rat BDNF genes consists of at least 8 distinct 5′ exons and one common 3′ exon (exon IX) that contains the coding region (Aid et al., 2007). Among those exons, promoter IV driven BDNF mRNA was regarded as the neural activity-dependent transcripts (Hong et al., 2008; Yasuda et al., 2009), which corresponds to exon/promoter III in older studies (Metsis et al., 1993; Timmusk and Metsis, 1994). So we checked BDNF mRNA level using primers directed to promoter IV. Interestingly, treatment of bicuculline also increased phosphorylation of both ERK and CREB (Fig. 3C), which suggest that $GABA_A$ antagonistic property of oroxylin A is involved

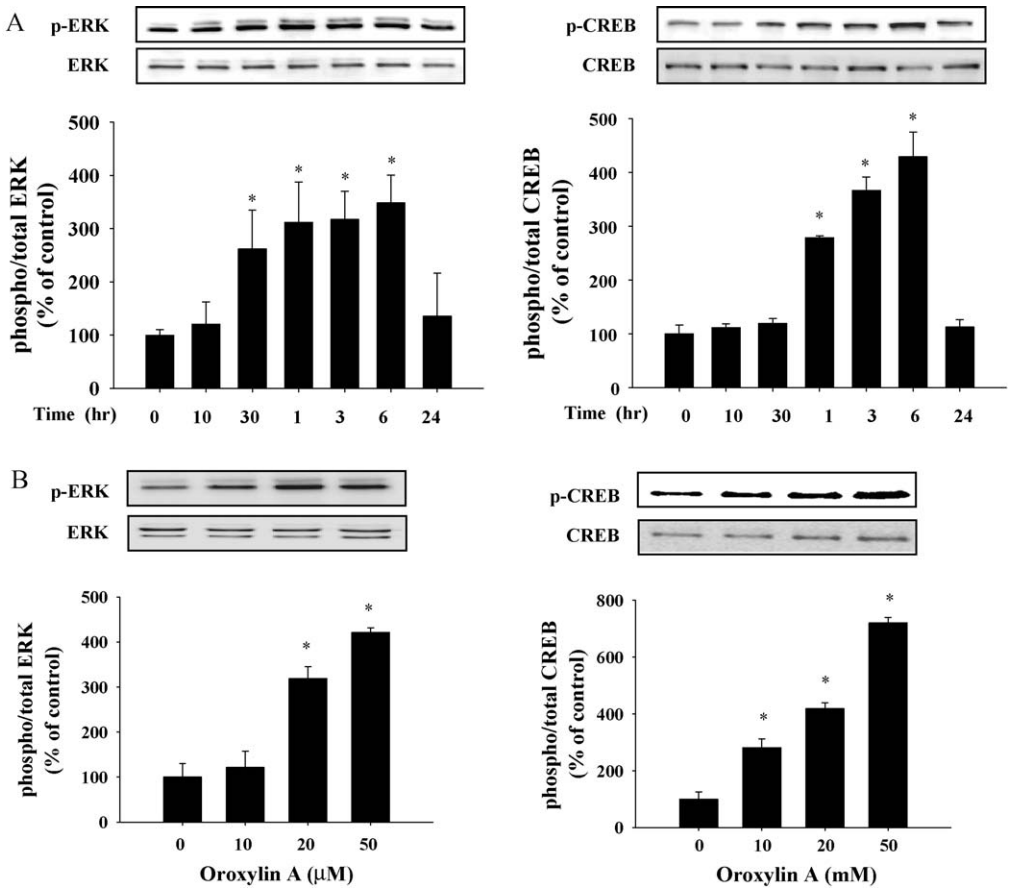

Fig. 2. Oroxylin A phosphorylated ERK and CREB in time- and concentration-dependent manner. (A) Oroxylin A (20 μM) was treated for various time points (10 min–24 h) in cortical neuronal culture and phosphorylation of ERK and CREB was analyzed by Western blot. (B) Alternatively, various concentrations of oroxylin A (10–50 μM, 1 h for ERK, 6 h for CREB) were treated before Western blot. Graphs represent the densitometric quantification of the band intensity. $^*p < 0.01$ versus untreated control value ($n = 9$).

in the phosphorylation of ERK and CREB followed by the induction of BDNF expression and release in rat primary cortical neuronal culture. To support this hypothesis, $GABA_A$ agonist muscimol was treated before oroxylin A treatment and the phosphorylation of CREB was measured using Western blot (Fig. 3D). Muscimol alone did not alter CREB phosphorylation status, however, it reduced the oroxylin A-induced CREB phosphorylation in a concentration dependent manner. These results suggest that the inhibition of $GABA_A$ receptor by oroxylin A triggered phosphorylation of ERK and CREB as well as BDNF production in cortical neuronal culture system.

To investigate the causal relationship of ERK1/2 phosphorylation and CREB activation, we used PD98059, a MEK inhibitor, to inhibit ERK1/2 phosphorylation. PD98059 (10 μM) was pretreated for 30 min before 1 h treatment of oroxylin A. Oroxylin A-induced phosphorylation of ERK1/2 was inhibited by PD98059 (Fig. 4A). At 6 h, the phosphorylation of CREB was also inhibited by PD98059 (Fig. 4A) implicating that oroxylin A phosphorylates CREB through the activation of MAPK pathway. In addition, the increased release of BDNF by oroxylin A or bicuculline was blocked by pretreatment of PD98059 (Fig. 4B). These results suggest that $GABA_A$ antagonistic property of oroxylin A regulates ERK1/2 phosphorylation, which again phosphorylates and activates CREB, resulting in the increased production of BDNF in rat primary cortical neuronal culture system.

Since inhibition of endogenous GABA transmission may lead to the activation of synaptic NMDA receptor, which might lead to the increased intracellular calcium concentration as well as ERK phosphorylation (Arnold et al., 2005; Kozinn et al., 2006; Doyle et al., 2010), we determined whether the activation of NMDA receptor is involved in the oroxylin A-induced BDNF expression. In this study, we pretreated a NMDA receptor inhibitor, MK-801, for 30 min before the treatment of oroxylin A. The phosphorylation of ERK1/2 and CREB by oroxylin A was effectively inhibited by MK-801 (Fig. 5A) as well as BDNF mRNA (Fig. 5B) and protein (Fig. 5C) expression. In addition, the increased release of BDNF by oroxylin A was inhibited by MK801 (Fig. 5D). Finally, we examined whether oroxylin A induces increase in intracellular calcium concentration. Treatment of oroxylin A induced slow but sustained increase in intracellular calcium concentration, although the extent of calcium increase was a little bit lower compared with a calcium ionophore ionomycin (Fig. 6A). Interestingly, the rise in intracellular calcium concentration was prevented by preincubation of MK-801 suggesting that the activation of NMDA receptor mediates the increase in intracellular calcium concentration by oroxylin A in rat primary cortical neurons. Also voltage-gated sodium channel blocker, TTX pretreatment reduced oroxylin A-induced increase in intracellular calcium concentration (80.52% compared to oroxylin A treated samples) (Fig. 6A). Similarly, BDNF production was inhibited following TTX and oroxylin A treatment compared with oroxylin A alone (0.5 μM of TTX: 2.03 ± 1.21-fold decrease, 1 μM of TTX: 4.14 ± 2.31-fold of decrease, respectively) (Fig. 6B), suggesting the increased neurotransmission is essential in the oroxylin A-mediated upregulation of BDNF in our cortical culture system.

4. Discussion

In previous studies, we have reported that oroxylin A, a flavonoid compound from *S. baicalensis* Georgi, ameliorates memory dysfunc-

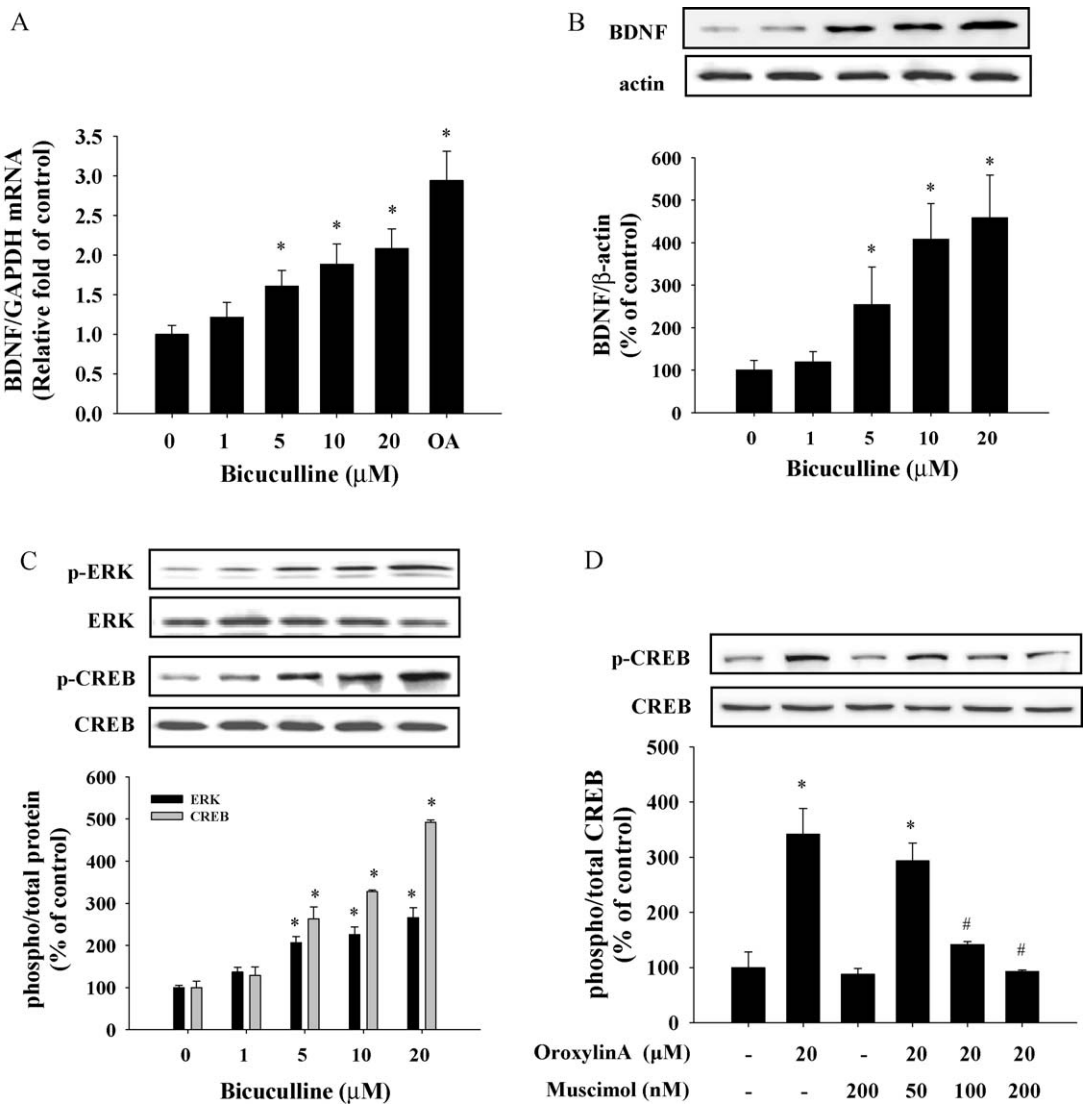

Fig. 3. Bicuculine increased expression of BDNF as well as ERK and CREB phosphorylation. A $GABA_A$ receptor antagonist bicuculline was treated for 24 h in rat primary cortical neuronal culture. BDNF mRNA expression (A) and protein expression (B) was determined by real time RT-PCR and Western blot, respectively. The phosphorylation of ERK and CREB (C) was analyzed by Western blot. (D) A $GABA_A$ receptor agonist muscimol was pretreated at 50–200 nM concentrations for 30 min followed by oroxylin A treatment. After 6 h incubation, CREB phosphorylation was analyzed using Western blot. Graphs represent the densitometric quantification of the band intensity. $^*p < 0.01$ versus untreated control value, and $^\#p < 0.01$ versus oroxylin A treated samples ($n = 3$).

tion induced by scopolamine, hypoperfusion induced by 2VO, and microinjection of Aβ (25–35) (Kim et al., 2006, 2007, 2008). We also reported that oroxylin A showed anti-inflammatory and neuroprotective action. In the hippocampal CA1 and dentate gyrus regions of 2-VO mice, increased CREB phosphorylation and BDNF positive neurons have been found suggesting oroxylin A may increase BDNF expression, which plays critical role in the regulation of neuronal development, synaptic plasticity and protection against neural damage.

BDNF provides protective effect on neurons through binding to TrkB and p75 receptor (Miyata et al., 2001; Gooney et al., 2002; Meng et al., 2005). Binding of BDNF to these receptors activates ERK and PI3K pathways and exerts surviving effects (Hetman et al., 1999; Han and Holtzman, 2000; Mograbi et al., 2001; Nakazawa et al., 2002).

In this study, we investigated whether oroxylin A regulates BDNF expression and release in cultured rat primary neurons as well as the intracellular signaling mechanism by which oroxylin A modulates BDNF expression. Our results showed that oroxylin A increased BDNF expression and release by sequential activation of ERK1/2 and CREB, which is mediated, at least in part, by the activation of NMDA receptor and increase in intracellular calcium concentration. The activation of the NMDA-ERK1/2-CREB pathway seems to be modulated by the $GABA_A$ antagonistic properties of oroxylin A, possibly via increased neuronal activity as evidenced by the inhibitory action of TTX.

It has been reported that neural activity modulates BDNF secretion as well as transcription and translation (Lu, 2003). Binding of BDNF to TrkB led to homo-dimerization and autophosphorylation of the receptors triggering the activation of three major signaling cascades including Ras-ERK MAPK, phospholipase C-γ (PLC-γ) and phosphatidylinositol-3 kinase (PI3K) pathways (Lu, 2003). Ras-ERK pathways lead to CREB phosphorylation and expression of CRE-dependent genes like BDNF and Arc (Ying et al., 2002). This auto-regulatory loop would facilitate BDNF transcription and release from neuronal circuits. Whether oroxylin A induces direct release of pre-existing BDNF in our culture condition should be investigated further in the future study.

Oroxylin A induced BDNF upregulation in astrocytes (Fig. 1) as well as in neuron. There have been many reports about the

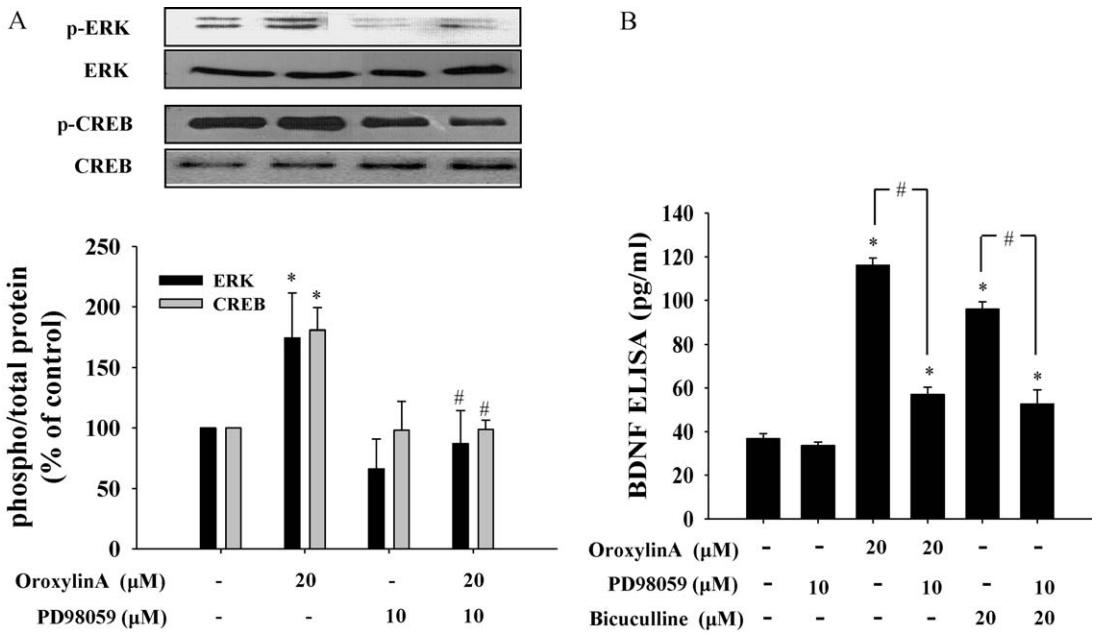

Fig. 4. Role of MAPK pathway activation on CREB phosphorylation and BDNF production. (A) Oroxylin A (20 μM) was treated after pretreatment of a MEK inhibitor, PD98059 (10 μM) for 30 min. Phosphorylation of ERK and CREB was analyzed by Western blot. PD98059 inhibited both ERK and CREB phosphorylation. Graphs represent the densitometric quantification of the band intensity. (B) Oroxylin A or bicuculline (20 μM) was treated for 24 h in the presence of a MEK inhibitor, PD98059 (10 μM) and BDNF release was determined by ELISA in culture supernatants as described in Section 2. $^{*}p < 0.01$ versus untreated control value. $^{\#}p < 0.01$ versus oroxylin A or bicuculline treated group ($n = 3$).

existence of $GABA_A$ receptors in cultured astrocytes (Kettenmann et al., 1987; Ventimiglia et al., 1990) and secretion/production of BDNF is regulated by several neuromodulators/neurotransmitters such as prostaglandins (Toyomoto et al., 2004), dopamine agonists including bromocriptine, pergolide, cabergoline, and SKF-38393 (Ohta et al., 2003) as well as other clinical and experimental drugs (Savli et al., 2004). Unlike the situation in neuronal circuitry, no clear mechanism has been suggested so far to correlate the inhibition of GABA receptor with increased expression of BDNF in astrocyte, although it is possible that small amount of contaminating neurons (1–2% in our culture condition) may induce calcium oscillation in cultured astrocytes in activity-dependent manner (Hirase et al., 2004). Another possibility is that oroxylin A may regulate different receptor systems in astrocytes, either directly or indirectly. Our unpublished results suggest that oroxylin A has moderate affinity for adenosine receptor subtypes (data not shown). In any cases, BDNF participates in astrocyte and neuronal communication throughout developmental stage (Ortega and Alcantara, 2010) as well as post maturation period for proper brain function (Rudge et al., 1995; Reichardt, 2003). Considering the pivotal role of astrocyte in the maintenance of proper environment for optimal neurotransmission, elucidating the actions of oroxylin A on BDNF secretion/production and its physiological roles in astrocytes–neuron interaction is an important and interesting issue to be resolved in the future study.

Blocking endogenous GABA activity using bicuculline has been used to stimulate synaptic NMDA activation (Hardingham et al., 2002). It is believed that the application of bicuculline induces the release of synaptic glutamate by removing inhibitory action of GABA thus inducing glutamate-driven activity of the neuronal network (Hardingham et al., 2002; Ivanov et al., 2006). In 14 DIV hippocampal neuronal culture, brief application of bicuculline induced increase of the frequency of spontaneous excitatory postsynaptic currents (EPSCs), which results in a rapid and strong increase of ERK phosphorylation (Payne et al., 1991; Ivanov et al., 2006). Interestingly, the bicuculline-induced ERK phosphorylation was effectively prevented by MK-801 suggesting the essential role of NMDA activation on $GABA_A$ blockade-mediated activation of ERK pathway (Ivanov et al., 2006). At this moment, it should be noted that depending on the context of glutamatergic neuronal stimulation, activation of NMDA receptor system may confer both physiological and pathological outcomes. Weak or modest extent of activation of NMDA receptor, possibly via synaptic activation of NMDA receptor with weak rise in intracellular calcium concentration (Kim et al., 2005), has been implicated in LTP induction (Lu et al., 2001), CREB activation as well as neuroprotection against neuronal insults (Hardingham et al., 2002). In contrast, strong activation of NMDA system, possibly via the activation of extrasynaptic NMDA receptor by both application of high concentration of NMDA receptor agonist with a strong increase of intracellular calcium, has been implicated in excitotoxic neuronal cell death (Hardingham et al., 2002). Similar to these findings, it has been suggested that that activation of NMDA receptors may exert bidirectional control of ERK activity with bell shaped concentration–response relationship (Chandler et al., 2001; Kim et al., 2005). Considering the mode of action of oroxylin A ($GABA_A$ antagonism) and weak rise in intracellular calcium concentration, oroxylin A-induced NMDA activation may provide neuroprotection and synaptic plasticity via mechanism, at least in part, involving ERK-CREB-BDNF activation in neuronal culture. We are under active investigation of the possible role and mechanism of oroxylin A in neuroprotection.

One of the most important regulators of the transcriptional activation of BDNF expression is the phosphorylation of CREB, which can be regulated by various signaling cascades including MAPK/ERK pathway and increase in intracellular Ca^{2+} concentration (Dash et al., 1991; Sheng et al., 1991; Xing et al., 1998). In addition to its well known role in a variety of cellular processes, for example, cell survival, cell proliferation and gene transcription, rise in intracellular calcium concentration has been implicated in the regulation of learning and memory process. Regulation of intracellular calcium concentration is involved in long term potentiation (LTP) and spatial memory which are impaired when signaling through NMDA receptor was blocked (Lynch et al., 1983; Davis et al., 1992). Increased intracellular calcium is also involved in the phosphoryla-

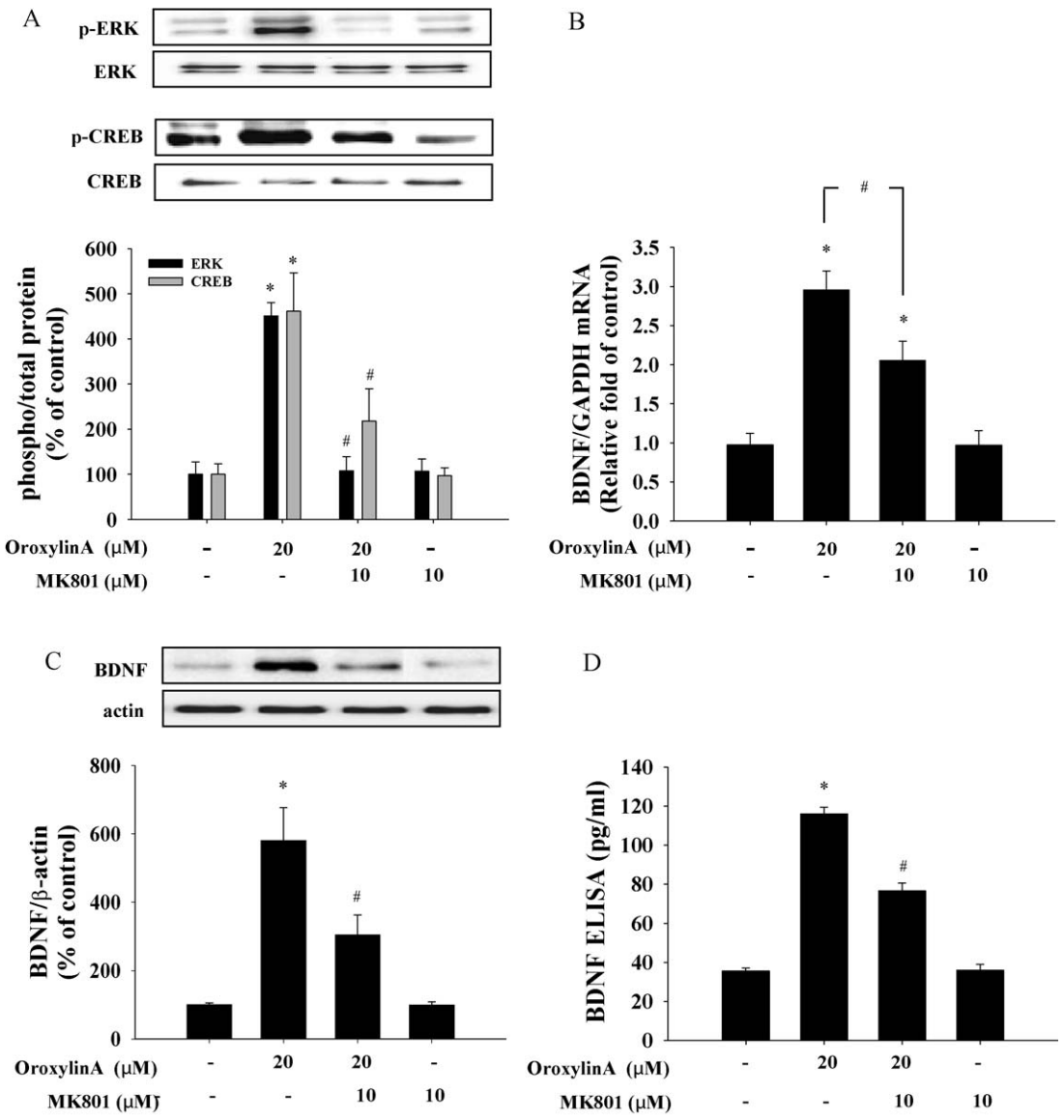

Fig. 5. Essential role of NMDA receptor activation on oroxylin A-induced ERK-CREB phosphorylation and BDNF expression. Oroxylin A (20 μM) was treated on cortical neuronal culture after pretreatment of a NMDA receptor antagonist MK801 (10 μM) for 30 min. (A) Phosphorylation of ERK and CREB was analyzed by Western blot. MK801 inhibited both ERK and CREB phosphorylation. Graphs represent the densitometric quantification of the band intensity. Similarly, the expression of BDNF mRNA (B), protein (C) as well as BDNF release (D) was determined by real time RT-PCR, Western blot and ELISA as above. $^{*}p < 0.01$versus untreated control value. $^{\#}p < 0.01$ versus oroxylin A treated group ($n = 9$).

tion of CREB. In addition to the regulation of classical intracellular signaling pathways, which may related to the increased phosphorylation of CREB, increased intracellular Ca^{2+} induces nuclear Ca^{2+} influx and activates Ca^{2+}/calmodulin kinase IV (CAMK IV) that is localized in nuclei of brain cells (Watanabe et al., 1996; Zubrow et al., 2002). Activation of CAMK IV results in phosphorylation of CREB (Soderling, 1999). Calcium is involved in regulating gene transcription not only by phosphorylating CREB, but also by activating CBP that is co-transcription factor with CREB (Chawla et al., 1998). Using sensitive calcium indicator, Fluo3/AM we analyzed changes of intracellular calcium. The results from this study show oroxylin A induced modest but sustained increase in intracellular Ca^{2+}, which was inhibited by NMDA receptor blocker, MK-801. MK-801 binds to the ion channel of NMDA receptor and prevents flow of ions such as calcium (Wong et al., 1986). Since, CBP can be activated by increase in nuclear calcium (Chawla et al., 1998), whether regulation of BDNF expression by oroxylin A also involves activation of CBP would be another point of concern to be examined in the future study.

$GABA_A$ receptor antagonistic properties of oroxylin A suggest that it may activate cultured cortical neurons by inducing glutamate release, which mediates activation of synaptic NMDA receptors (Ikeda et al., 2008) as in the case of bicuculline. In this study, TTX treatment, which inhibits progression of action potential in neural circuits prevented oroxylin A-induced increase in intracellular calcium concentration as well as BDNF induction suggesting the importance of the modulation of neurotransmission in oroxylin-A induced upregulation of BDNF.

Neuronal activity dependent release and expression of BDNF is important for reorganization of synaptic connectivity in the CNS. Especially, BDNF has pleiotropic effects on CNS function, for example, neuronal development, synaptic plasticity such as cognition, memory, learning, neuronal structure, mood and behavior (Greenberg et al., 2009). Cells respond to neuronal activation

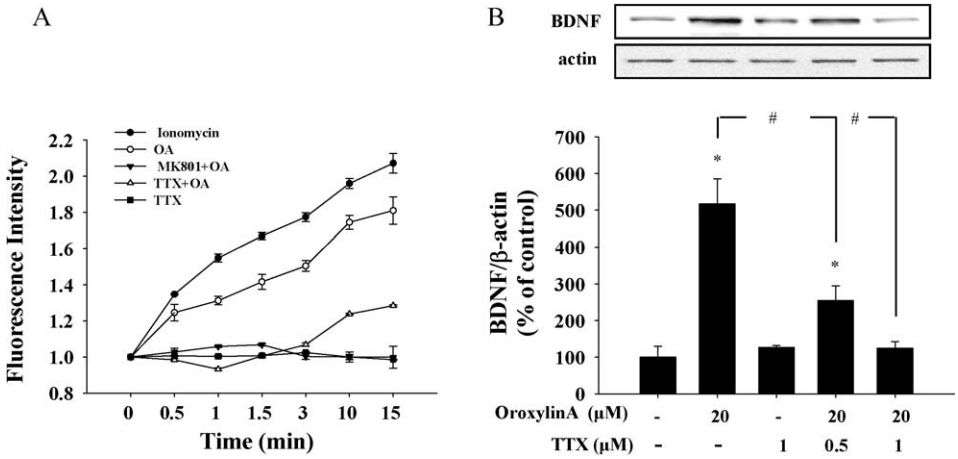

Fig. 6. Increased intracellular calcium concentration and BDNF expression by oroxylin A is regulated by the modulation of neurotransmission. (A) Intracellular Ca^{2+} level was observed using Fluo3-AM for 20 min as described in Section 2 after treatment of oroxylin A (20 μM), and ionomycin (10 μM, a positive control). Oroxylin A induced slow and sustained increase in intracellular Ca^{2+} level. Pre-incubation of a NMDA receptor inhibitor, MK-801 (10 μM, 15 min), and sodium channel blocker tetrodotoxin (1 μM, 30 min) blocked increase in intracellular Ca^{2+} level induced by oroxylin A ($n = 3$). (B) After TTX pretreatment (0.5–1 μM, 30 min), oroxylin A was treated on cultured cortical neurons. Then BDNF protein induction was analyzed by Western blot. Graphs represent the densitometric quantification of the band intensity. $^{*}p < 0.01$ versus untreated control value, and $^{\#}p < 0.01$ versus oroxylin A treated samples ($n = 3$).

by rapidly upregulating promoter IV-driven BDNF transcription (Timmusk and Metsis, 1994). Initially, BDNF is synthesized as pre-pro BDNF in endoplasmic reticulum and cleaved to pro BDNF (Gray and Ellis, 2008; Matsumoto et al., 2008). These pro BDNF can be converted to mature BDNF by plasmin through the tissue plasminogen activator (tPA) system, which is related with long term potentiation (LTP) and memory (Greenberg et al., 2009). Pro form may modulate long term depression (LTD) through p75 receptor in adult hippocampus, while high frequency stimulation leads to tPA secretion which can convert pro BDNF to mature BDNF, that regulates LTP through Trk B receptor. Since impairments in these regulatory systems are connected to neuropsychiatric disorders, depression, circuit abnormalities, and behavioral problems, it implies the importance of continued study into the fine tuning of neuronal activity-regulated genes, such as BDNF, and the synaptic signaling pathways which regulate their expression.

In summary, a neuroprotective and memory-enhancing flavonoid oroxylin A regulated BDNF expression by NMDA-ERK–CREB pathway and/or increased intracellular calcium concentration through its effects as a $GABA_A$ antagonist. Investigating whether the increased BDNF expression by oroxylin A can provide actual neuroprotective roles in various neurological insult conditions as well as in the regulation of synaptic plasticity both in terms of structural and functional aspects, would be valuable to establish new therapeutic potential of oroxylin A against various neurodegenerative and neuropsychological disorders.

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References

Aid, T., Kazantseva, A., Piirsoo, M., Palm, K., Timmusk, T., 2007. Mouse and rat BDNF gene structure and expression revisited. J. Neurosci. Res. 85, 525–535.

Arnold, F.J., Hofmann, F., Bengtson, C.P., Wittmann, M., Vanhoutte, P., Bading, H., 2005. Microelectrode array recordings of cultured hippocampal networks reveal a simple model for transcription and protein synthesis-dependent plasticity. J. Physiol. 564, 3–19.

Bekinschtein, P., Cammarota, M., Katche, C., Slipczuk, L., Rossato, J.I., Goldin, A., Izquierdo, I., Medina, J.H., 2008. BDNF is essential to promote persistence of long-term memory storage. Proc. Natl. Acad. Sci. U.S.A. 105, 2711–2716.

Brewer, G.J., Torricelli, J.R., Evege, E.K., Price, P.J., 1993. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. J. Neurosci. Res. 35, 567–576.

Chandler, L.J., Sutton, G., Dorairaj, N.R., Norwood, D., 2001. N-methyl D-aspartate receptor-mediated bidirectional control of extracellular signal-regulated kinase activity in cortical neuronal cultures. J. Biol. Chem. 276, 2627–2636.

Chawla, S., Hardingham, G.E., Quinn, D.R., Bading, H., 1998. CBP: a signal-regulated transcriptional coactivator controlled by nuclear calcium and CaM kinase IV. Science 281, 1505–1509.

Chen, Y., Yang, L., Lee, T.J., 2000. Oroxylin A inhibition of lipopolysaccharide-induced iNOS and COX-2 gene expression via suppression of nuclear factor-kappaB activation. Biochem. Pharmacol. 59, 1445–1457.

Croll, S.D., Wiegand, S.J., Anderson, K.D., Lindsay, R.M., Nawa, H., 1994. Regulation of neuropeptides in adult rat forebrain by the neurotrophins BDNF and NGF. Eur. J. Neurosci. 6, 1343–1353.

Dash, P.K., Karl, K.A., Colicos, M.A., Prywes, R., Kandel, E.R., 1991. cAMP response element-binding protein is activated by Ca2+/calmodulin– as well as cAMP-dependent protein kinase. Proc. Natl. Acad. Sci. U.S.A. 88, 5061–5065.

Davis, S., Butcher, S.P., Morris, R.G., 1992. The NMDA receptor antagonist D-2-amino-5-phosphonopentanoate (D-AP5) impairs spatial learning and LTP in vivo at intracerebral concentrations comparable to those that block LTP in vitro. J. Neurosci. 12, 21–34.

Doyle, S., Pyndiah, S., De Gois, S., Erickson, J.D., 2010. Excitation-transcription coupling via calcium/calmodulin-dependent protein kinase/ERK1/2 signaling mediates the coordinate induction of VGLUT2 and Narp triggered by a prolonged increase in glutamatergic synaptic activity. J. Biol. Chem. 285, 14366–14376.

Gooney, M., Shaw, K., Kelly, A., O'Mara, S.M., Lynch, M.A., 2002. Long-term potentiation and spatial learning are associated with increased phosphorylation of TrkB and extracellular signal-regulated kinase (ERK) in the dentate gyrus: evidence for a role for brain-derived neurotrophic factor. Behav. Neurosci. 116, 455–463.

Gray, K., Ellis, V., 2008. Activation of pro-BDNF by the pericellular serine protease plasmin. FEBS Lett. 582, 907–910.

Greenberg, M.E., Xu, B., Lu, B., Hempstead, B.L., 2009. New insights in the biology of BDNF synthesis and release: implications in CNS function. J. Neurosci. 29, 12764–12767.

Han, B.H., Holtzman, D.M., 2000. BDNF protects the neonatal brain from hypoxic–ischemic injury in vivo via the ERK pathway. J. Neurosci. 20, 5775–5781.

Hardingham, G.E., Fukunaga, Y., Bading, H., 2002. Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. Nat. Neurosci. 5, 405–414.

Hetman, M., Kanning, K., Cavanaugh, J.E., Xia, Z., 1999. Neuroprotection by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol 3-kinase. J. Biol. Chem. 274, 22569–22580.

Hirase, H., Qian, L., Bartho, P., Buzsaki, G., 2004. Calcium dynamics of cortical astrocytic networks in vivo. PLoS Biol. 2, E96.

Hong, E.J., McCord, A.E., Greenberg, M.E., 2008. A biological function for the neuronal activity-dependent component of Bdnf transcription in the development of cortical inhibition. Neuron 60, 610–624.

Huen, M.S., Hui, K.M., Leung, J.W., Sigel, E., Baur, R., Wong, J.T., Xue, H., 2003a. Naturally occurring 2′-hydroxyl-substituted flavonoids as high-affinity benzodiazepine site ligands. Biochem. Pharmacol. 66, 2397–2407.

Huen, M.S., Leung, J.W., Ng, W., Lui, W.S., Chan, M.N., Wong, J.T., Xue, H., 2003b. 5,7-Dihydroxy-6-methoxyflavone, a benzodiazepine site ligand isolated from *Scutellaria baicalensis* Georgi, with selective antagonistic properties. Biochem. Pharmacol. 66, 125–132.

Ikeda, H., Kiritoshi, T., Murase, K., 2008. Effect of excitatory and inhibitory agents and a glial inhibitor on optically-recorded primary-afferent excitation. Mol. Pain 4, 39.

Impey, S., McCorkle, S.R., Cha-Molstad, H., Dwyer, J.M., Yochum, G.S., Boss, J.M., McWeeney, S., Dunn, J.J., Mandel, G., Goodman, R.H., 2004. Defining the CREB regulon: a genome-wide analysis of transcription factor regulatory regions. Cell 119, 1041–1054.

Ip, N.Y., Li, Y., Yancopoulos, G.D., Lindsay, R.M., 1993. Cultured hippocampal neurons show responses to BDNF, NT-3, and NT-4, but not NGF. J. Neurosci. 13, 3394–3405.

Ivanov, A., Pellegrino, C., Rama, S., Dumalska, I., Salyha, Y., Ben-Ari, Y., Medina, I., 2006. Opposing role of synaptic and extrasynaptic NMDA receptors in regulation of the extracellular signal-regulated kinases (ERK) activity in cultured rat hippocampal neurons. J. Physiol. 572, 789–798.

Jiwajinda, S., Santisopasri, V., Murakami, A., Kim, O.K., Kim, H.W., Ohigashi, H., 2002. Suppressive effects of edible Thai plants on superoxide and nitric oxide generation. Asian Pac. J. Cancer Prev. 3, 215–223.

Kettenmann, H., Backus, K.H., Schachner, M., 1987. gamma-Aminobutyric acid opens Cl-channels in cultured astrocytes. Brain Res. 404, 1–9.

Kim, D.H., Jeon, S.J., Son, K.H., Jung, J.W., Lee, S., Yoon, B.H., Choi, J.W., Cheong, J.H., Ko, K.H., Ryu, J.H., 2006. Effect of the flavonoid, oroxylin A, on transient cerebral hypoperfusion-induced memory impairment in mice. Pharmacol. Biochem. Behav. 85, 658–668.

Kim, D.H., Jeon, S.J., Son, K.H., Jung, J.W., Lee, S., Yoon, B.H., Lee, J.J., Cho, Y.W., Cheong, J.H., Ko, K.H., Ryu, J.H., 2007. The ameliorating effect of oroxylin A on scopolamine-induced memory impairment in mice. Neurobiol. Learn. Mem. 87, 536–546.

Kim, D.H., Kim, S., Jeon, S.J., Son, K.H., Lee, S., Yoon, B.H., Cheong, J.H., Ko, K.H., Ryu, J.H., 2008. The effects of acute and repeated oroxylin A treatments on Abeta(25–35)-induced memory impairment in mice. Neuropharmacology 55, 639–647.

Kim, M.J., Dunah, A.W., Wang, Y.T., Sheng, M., 2005. Differential roles of NR2A- and NR2B-containing NMDA receptors in Ras-ERK signaling and AMPA receptor trafficking. Neuron 46, 745–760.

Kozinn, J., Mao, L., Arora, A., Yang, L., Fibuch, E.E., Wang, J.Q., 2006. Inhibition of glutamatergic activation of extracellular signal-regulated protein kinases in hippocampal neurons by the intravenous anesthetic propofol. Anesthesiology 105, 1182–1191.

Kwok, R.P., Lundblad, J.R., Chrivia, J.C., Richards, J.P., Bachinger, H.P., Brennan, R.G., Roberts, S.G., Green, M.R., Goodman, R.H., 1994. Nuclear protein CBP is a coactivator for the transcription factor CREB. Nature 370, 223–226.

Lewin, G.R., 1996. Neurotrophins and the specification of neuronal phenotype. Philos. Trans. R. Soc. Lond. B: Biol. Sci. 351, 405–411.

Lindholm, D., Dechant, G., Heisenberg, C.P., Thoenen, H., 1993. Brain-derived neurotrophic factor is a survival factor for cultured rat cerebellar granule neurons and protects them against glutamate-induced neurotoxicity. Eur. J. Neurosci. 5, 1455–1464.

Lu, B., 2003. BDNF and activity-dependent synaptic modulation. Learn. Mem. 10, 86–98.

Lu, W., Man, H., Ju, W., Trimble, W.S., MacDonald, J.F., Wang, Y.T., 2001. Activation of synaptic NMDA receptors induces membrane insertion of new AMPA receptors and LTP in cultured hippocampal neurons. Neuron 29, 243–254.

Lynch, G., Larson, J., Kelso, S., Barrionuevo, G., Schottler, F., 1983. Intracellular injections of EGTA block induction of hippocampal long-term potentiation. Nature 305, 719–721.

Marty, S., Carroll, P., Cellerino, A., Castren, E., Staiger, V., Thoenen, H., Lindholm, D., 1996. Brain-derived neurotrophic factor promotes the differentiation of various hippocampal nonpyramidal neurons, including Cajal-Retzius cells, in organotypic slice cultures. J. Neurosci. 16, 675–687.

Matsumoto, T., Rauskolb, S., Polack, M., Klose, J., Kolbeck, R., Korte, M., Barde, Y.A., 2008. Biosynthesis and processing of endogenous BDNF: CNS neurons store and secrete BDNF, not pro-BDNF. Nat. Neurosci. 11, 131–133.

Meng, M., Zhiling, W., Hui, Z., Shengfu, L., Dan, Y., Jiping, H., 2005. Cellular levels of TrkB and MAPK in the neuroprotective role of BDNF for embryonic rat cortical neurons against hypoxia in vitro. Int. J. Dev. Neurosci. 23, 515–521.

Metsis, M., Timmusk, T., Arenas, E., Persson, H., 1993. Differential usage of multiple brain-derived neurotrophic factor promoters in the rat brain following neuronal activation. Proc. Natl. Acad. Sci. U.S.A. 90, 8802–8806.

Miyata, K., Omori, N., Uchino, H., Yamaguchi, T., Isshiki, A., Shibasaki, F., 2001. Involvement of the brain-derived neurotrophic factor/TrkB pathway in neuroprotecive effect of cyclosporin A in forebrain ischemia. Neuroscience 105, 571–578.

Mograbi, B., Bocciardi, R., Bourget, I., Busca, R., Rochet, N., Farahi-Far, D., Juhel, T., Rossi, B., 2001. Glial cell line-derived neurotrophic factor-stimulated phosphatidylinositol 3-kinase and Akt activities exert opposing effects on the ERK pathway: importance for the rescue of neuroectodermic cells. J. Biol. Chem. 276, 45307–45319.

Nakazawa, T., Tamai, M., Mori, N., 2002. Brain-derived neurotrophic factor prevents axotomized retinal ganglion cell death through MAPK and PI3K signaling pathways. Invest. Ophthalmol. Vis. Sci. 43, 3319–3326.

Nawa, H., Pelleymounter, M.A., Carnahan, J., 1994. Intraventricular administration of BDNF increases neuropeptide expression in newborn rat brain. J. Neurosci. 14, 3751–3765.

Ohta, K., Kuno, S., Mizuta, I., Fujinami, A., Matsui, H., Ohta, M., 2003. Effects of dopamine agonists bromocriptine, pergolide, cabergoline, and SKF-38393 on GDNF, NGF, and BDNF synthesis in cultured mouse astrocytes. Life Sci. 73, 617–626.

Ortega, J.A., Alcantara, S., 2010. BDNF/MAPK/ERK-induced BMP7 expression in the developing cerebral cortex induces premature radial glia differentiation and impairs neuronal migration. Cereb. Cortex 20, 2132–2144.

Park, G.H., Jeon, S.J., Ryu, J.R., Choi, M.S., Han, S.H., Yang, S.I., Ryu, J.H., Cheong, J.H., Shin, C.Y., Ko, K.H., 2009. Essential role of mitogen-activated protein kinase pathways in protease activated receptor 2-mediated nitric-oxide production from rat primary astrocytes. Nitric Oxide 21, 110–119.

Payne, D.M., Rossomando, A.J., Martino, P., Erickson, A.K., Her, J.H., Shabanowitz, J., Hunt, D.F., Weber, M.J., Sturgill, T.W., 1991. Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). EMBO J. 10, 885–892.

Reichardt, L.F., 2003. Neurobiology: signals that make waves. Nature 426, 25–26.

Rudge, J.S., Pasnikowski, E.M., Holst, P., Lindsay, R.M., 1995. Changes in neurotrophic factor expression and receptor activation following exposure of hippocampal neuron/astrocyte cocultures to kainic acid. J. Neurosci. 15, 6856–6867.

Savli, H., Gulkac, M.D., Esen, N., 2004. The effect of stimulated microglia conditioned media on BDNF gene expression of striatal astrocytes: quantification by real-time PCR. Int. J. Neurosci. 114, 1601–1612.

Shaywitz, A.J., Greenberg, M.E., 1999. CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. Annu. Rev. Biochem. 68, 821–861.

Sheng, M., Thompson, M.A., Greenberg, M.E., 1991. CREB: a Ca(2+)-regulated transcription factor phosphorylated by calmodulin-dependent kinases. Science 252, 1427–1430.

Soderling, T.R., 1999. The Ca-calmodulin-dependent protein kinase cascade. Trends Biochem. Sci. 24, 232–236.

Tao, X., Finkbeiner, S., Arnold, D.B., Shaywitz, A.J., Greenberg, M.E., 1998. Ca2+ influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism. Neuron 20, 709–726.

Timmusk, T., Metsis, M., 1994. Regulation of BDNF promoters in the rat hippocampus. Neurochem. Int. 25, 11–15.

Toyomoto, M., Ohta, M., Okumura, K., Yano, H., Matsumoto, K., Inoue, S., Hayashi, K., Ikeda, K., 2004. Prostaglandins are powerful inducers of NGF and BDNF production in mouse astrocyte cultures. FEBS Lett. 562, 211–215.

Ventimiglia, R., Grierson, J.P., Gallombardo, P., Sweetnam, P.M., Tallman, J.F., Geller, H.M., 1990. Cultured rat neurons and astrocytes express immunologically related epitopes of the $GABA_A$/benzodiazepine receptor. Neurosci. Lett. 115, 131–136.

Wang, Y.F., Tsirka, S.E., Strickland, S., Stieg, P.E., Soriano, S.G., Lipton, S.A., 1998. Tissue plasminogen activator (tPA) increases neuronal damage after focal cerebral ischemia in wild-type and tPA-deficient mice. Nat. Med. 4, 228–231.

Watanabe, S., Okuno, S., Kitani, T., Fujisawa, H., 1996. Inactivation of calmodulin-dependent protein kinase IV by autophosphorylation of serine 332 within the putative calmodulin-binding domain. J. Biol. Chem. 271, 6903–6910.

Wong, E.H., Kemp, J.A., Priestley, T., Knight, A.R., Woodruff, G.N., Iversen, L.L., 1986. The anticonvulsant MK-801 is a potent N-methyl-D-aspartate antagonist. Proc. Natl. Acad. Sci. U.S.A. 83, 7104–7108.

Xing, J., Kornhauser, J.M., Xia, Z., Thiele, E.A., Greenberg, M.E., 1998. Nerve growth factor activates extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways to stimulate CREB serine 133 phosphorylation. Mol. Cell Biol. 18, 1946–1955.

Yasuda, S., Liang, M.H., Marinova, Z., Yahyavi, A., Chuang, D.M., 2009. The mood stabilizers lithium and valproate selectively activate the promoter IV of brain-derived neurotrophic factor in neurons. Mol. Psychiatry 14, 51–59.

Ying, S.W., Futter, M., Rosenblum, K., Webber, M.J., Hunt, S.P., Bliss, T.V., Bramham, C.R., 2002. Brain-derived neurotrophic factor induces long-term potentiation in intact adult hippocampus: requirement for ERK activation coupled to CREB and upregulation of Arc synthesis. J. Neurosci. 22, 1532–1540.

Zubrow, A.B., Delivoria-Papadopoulos, M., Ashraf, Q.M., Fritz, K.I., Mishra, O.P., 2002. Nitric oxide-mediated Ca2+/calmodulin-dependent protein kinase IV activity during hypoxia in neuronal nuclei from newborn piglets. Neurosci. Lett. 335, 5–8.