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NMDA-mediated and Self-induced *Bdnf* Exon IV Transcriptions are Differentially Regulated in Cultured Cortical Neurons

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Abstract

Activity-dependent transcriptional up-regulation of *bdnf* (brain-derived neurotrophic factor) is involved in regulating many aspects of neuronal functions. The NMDA (N-methyl-D-aspartic acid)-mediated and BDNF-mediated *exon IV* transcription may represent mechanistically different responses, and relevant to activity-dependent changes in neurons. We found that the activities of ERK (extracellular signal-regulated kinase), CaM KII/IV (calmodulin-dependent protein kinase II and IV), PI3K (phosphoinositide 3-kinase), and PLC (phospholipase C) are required for NMDA receptor-mediated *bdnf exon IV* transcription in cultured cortical neurons. In contrast, the BDNF-induced and TrkB-dependent *exon IV* transcription was regulated by ERK and CaM KII/IV, but not by PI3K and PLC. While ERK and CaM KII/IV are separate signaling pathways in BDNF-stimulated neurons, CaM KII/IV appeared to regulate *exon IV* transcription through ERK in NMDA-stimulated neurons. Similarly, the PI3K and PLC signaling pathways converged on ERK in NMDA- but not BDNF-stimulated neurons. Our results implicate that the NMDA-induced and the self-maintenance of *bdnf* transcription are differentially regulated.

Keywords

NMDA; BDNF; transcription; CaM KII/IV; PI3K; ERK; PLC

1. Introduction

BDNF regulates neuroplasticity. Like other neurotrophins, BDNF was initially identified for its role in neuron proliferation, neurogenesis, differentiation and degeneration (Barde 1994; Connor and Dragunow 1998). Its function is also implicated in neurological disorders, such as bipolar disorder and Alzheimer's disease (Carter 2007a, b). However, its function in regulating synaptic plasticity has been convinced by numerous studies. BDNF has been implicated in regulating both memory formation and long-term potentiation (LTP), a cellular model for

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neuroplasticity (Malenka and Bear 2004). For example, BDNF knockout (KO) mice displayed deficits in LTP (Korte et al. 1995; Patterson et al. 1996) and spatial memory (Linnarsson et al. 1997). Furthermore, these defects in the KO mice were rescued by the virus-based over-expression of BDNF (Korte et al. 1996). Infusion of BDNF antisense oligonucleotides or anti-BDNF antibodies also impaired hippocampus-dependent memory (Ma et al. 1998; Mu et al. 1999). Studies on the mechanisms have indicated that BDNF-activated TrkB enhances glutamatergic synaptic transmission (Levine et al. 1995), increases the phosphorylation of NMDAR (Suen et al. 1997), and hence modulates LTP (Kovalchuk et al. 2002). In addition, BDNF/TrkB may modulate LTP at the pre-synaptic sites (Xu et al. 2000). The application of BDNF to cultured neurons increased glutamate release (Takei et al. 1998), possibly by enhancing vesicle docking at the active zone (Pozzo-Miller et al. 1999).

Interestingly, the expression of BDNF is tightly coupled to neuronal activity. First of all, kindling and kainic acid-treated brain showed dramatic increase in *bdnf* mRNA (Ernfors et al. 1991; Timmusk et al. 1993). Secondly, the induction of *bdnf* expression is observed in both LTP (Patterson et al. 1992) and memory formation (Ma et al. 1998; Hall et al. 2000). Previous studies demonstrated that the transcription of *bdnf* is regulated by Ca^{2+} -induced CREB and CaM KII/IV activation (Shieh et al. 1998; Tao et al. 1998; West et al. 2002). Genomic and cDNA sequence analysis of *bdnf* demonstrated that its transcription is controlled by eight promoters, leading to different transcripts containing one of the eight untranslated 5'-exons (I to VIII) and the 3' encoding exon (exon IX) (Timmusk et al. 1993; Timmusk et al. 1994; Aid et al. 2007). Among these eight promoters, the activity of promoter IV was strongly stimulated by Ca^{2+} (Tao et al. 1998; Tabuchi et al. 2000), leading to the up-regulation of exon IV-containing mRNA [initially designated as exon III, see (Timmusk et al. 1993)]. Molecular dissection of the promoter IV region identified cAMP responsive element (CRE) as the key regulatory component, implicating the function of the transcription factor CREB (CRE binding protein).

Recently, Yasuda et al. documented that *bdnf* expression could be stimulated by BDNF (Yasuda et al. 2007), suggesting a positive feed back mechanism for sustained transcriptional up-regulation of *bdnf*. It is conceivable that the initial up-regulation of *bdnf* transcription by neural activity through NMDA receptors and the subsequent self-stimulation may contribute to the induction and maintenance of *bdnf* expression, respectively. Additionally, NMDA stimulates both calcium influx and BDNF secretion (Kolarow et al. 2007). These two secondary events may concurrently activate *exon IV* transcription. Although both NMDA and BDNF stimulate CREB activation, it is not clear whether these stimulations share common mechanisms. In this study, we examined the signaling determinants for *bdnf exon IV* transcription in NMDA- and BDNF-stimulated neurons.

2. Materials and methods

2.1. Cell culture and treatments

Cultured cortical neurons were obtained from postnatal day 0 rats as described (Wang et al. 2003). Following dissection, cortices were chopped into small dices and digested with 10 units/ml papain in dissociation buffer (82 mM Na_2SO_4 , 30 mM K_2SO_4 , 5.8 mM MgCl_2 , 0.25 mM CaCl_2 , 20 mM glucose, 0.001% phenol red, and 1.5 mM HEPES, pH 7.6) at 37° C for 30 to 45 min. The digestion was washed and triturated. Approximately 0.5 million cells were seeded in 12-well plate coated with 100 $\mu\text{g}/\text{ml}$ poly-D-lysine. The cultures are maintained in Neurobasal A + B27 (Invitrogen). In vitro day (DIV) 6 to 8 neurons were stimulated by either NMDA (20 μM)/glycine (1 μM) or BDNF (5ng/ml) (EMD).

To block the activity of ERK, neurons were pre-treated with the MEK inhibitor U0126 (10 μM) for 30min before NMDA or BDNF stimulation. Similarly, the activity of CaM KII/IV was

blocked by KN93 (5uM). PI3K and PLC activity was inhibited by LY294002 (30uM) and U73122 (5uM), respectively. The activity of the Trk receptor tyrosine kinase was blocked by K252a (0.2uM) or TrkB-IgG (0.4ug/ml).

2.2. Polymerase Chain Reaction (PCR)

Total RNA was extracted from neurons 60min after the stimulation by the TRIzol methods (Invitrogen). Half microgram of total RNA was used for reverse transcription (RT). One tenth of RT product was then used for PCR amplification. Primers used for RT-PCR to amplify exons I-, II-, IV-, VI-containing *bdnf* and *GAPDH* mRNA (Tao et al. 1998; Tabuchi et al. 2000; Tabuchi et al. 2002) are listed in Table 1. According to the literature, the cycle number to amplify *exon I, II, IV, and VI* is 33, 31, 26, and 29, respectively (Tabuchi et al. 2000). The cycle number for *GAPDH* is 20. The annealing temperature is 55°C for all genes. The melting temperature is 94°C for all genes. The PCR products were separated by 1.2% agarose gel, and quantified using Scion Image software (Scion Corp. Frederick, Maryland)

The expression level of *exon IV*-containing *bdnf* was also examined by real time PCR, using Bio-Rad Syber Green System. PCR was performed with the same primers and annealing temperature, except that the cycle number was 40 to obtain the complete amplification curves. The concentration of cDNA was adjusted so that the threshold cycle was between 20 and 30 (data not shown). Each RT sample was analyzed in triplicate by the IQ5 software (Bio-Rad). The mRNA level was normalized to *GAPDH* and calculated with the $2^{-\Delta\Delta C_t}$ method.

2.3. Western blotting

Fifteen minutes after stimulation, samples were harvested in 50 ul SDS loading buffer (10mM Tris-HCl buffer pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate, 0.01% bromophenol blue and 5% β -mercaptoethanol). Lysates from 0.1 million neurons were separated by 10% SDS-PAGE, and transferred to nitrocellulose membranes. Antibodies against phospho-ERK1/2 (p-ERK) (1:1 000, Cell Signaling) were used to detect activated form of ERK. The incubation was overnight at 4°C for primary antibodies, and 1 hr at room temperature for secondary antibodies (HRP-conjugated, 1:5000, Pierce). Signal detection with the ECL system (SuperSignal® West Pico, Pierce, Rockford, IL) was used. The signal of p-ERK was normalized to the level of total ERK (determined by antibodies against total ERK, 1:1000, Cell Signaling), or beta-Actin (1:5000, Sigma). Several exposure times were used to obtain signals in the linear range. The signals were quantified using Scion Image software (Scion Corp. Frederick, Maryland). All quantification data are expressed as the average $\pm$ SEM. Differences with p-values less than 0.05 were considered significant.

2.4 Statistics

Comparison of two groups within several treatments was analyzed with post-hoc method (one-way ANOVA). All quantification data was presented as mean $\pm$ SEM. Difference was considered significant when p value is less than 0.05.

3. Results

3.1. NMDA and BDNF regulate mRNA transcription, but not stability, of *bdnf* exon IV

The activity-dependent gene transcription may be initiated by the calcium influx through NMDA receptors. Here, we confirmed that the exon IV-, but not exon I-, II, and VI-containing *bdnf* mRNA was significantly activated by NMDA stimulation in cultured cortical neurons (Fig. 1A). Blocking NMDA receptor by pre-treating neurons with APV abolished the up-regulation of *exon IV* transcription (Fig. 1A), indicating that the activation depends on NMDAR.

Theoretically, the increase in mRNA levels may be due to either increased transcription or decreased mRNA degradation. To rule out the function of NMDA receptor in regulating *bdnf* *exon IV* mRNA stability, we pre-treated neurons with a transcription inhibitor actinomycin (ACD). As shown in Fig. 1B, the level of *exon IV* was comparable between ACD-treated and ACD/NMDA-treated neurons, indicating that NMDA does not regulate mRNA stability. A recent study documented an interesting self stimulation of *bdnf* expression. Here, we demonstrated that BDNF specifically up-regulate the mRNA level of *exon IV*-, but not I-, II- and VI-containing transcripts in cultured cortical neurons (Fig. 1C). The dose responsive curve demonstrated that sub-nanomolar BDNF (5ng/ml or 0.15nM for BDNF dimer) lead to full scale activation of *exon IV*. The BDNF-mediated up-regulation of *exon IV* was abolished by K252a and TrkB-IgG, indicating the requirement of TrkB activation (Fig. 1D). Furthermore, similar levels of *exon IV* mRNA was observed for ACD- and ACD/BDNF-treated neurons, indicating that BDNF regulates transcription rather than mRNA stability (Fig. 1E).

3.2. Calmodulin-dependent protein kinases II and IV regulate NMDA-induced *bdnf* *exon IV* expression through the MAPK-ERK pathway

Previous molecular investigations demonstrated that CREB and CaM KII/IV are key regulators for the transcription of *bdnf*. It was also demonstrated that the activation of CREB requires the activity of both ERK and CaM KII. Consistently, by semi-quantitative RT-PCR, we found that NMDA-induced up-regulation of *exon IV* was blocked by both MEK inhibitor U0126 (left and middle panels of Fig. 2A) and CaM KII/IV inhibitor KN93 (left and middle panels of Fig. 2C). We further confirmed the NMDA-stimulated *exon IV* transcription, as well as the inhibitory effects of U0126 and KN93, by real-time PCR (right panels of Fig. 2A and C). Compared to the semi-quantitative method, real-time PCR was more sensitive, and detected more robust transcriptional up-regulation of *exon IV*. Semi-quantitative methods revealed an approximately 6-fold increase of *exon IV* in NMDA-treated neurons (middle panels, Fig. 2A and C). An average of 15-fold up-regulation was detected by the real-time PCR (right panels, Fig. 2A and C). When neurons were treated with KN92, the inactive analogue of KN93, NMDA-stimulated *exon IV* transcription was not blocked (right panel, Fig. 2C).

Interestingly, the NMDA-induced ERK phosphorylation was blocked by either U0126 or KN93 (Fig. 2B and 2D). These data demonstrated that CaM KII/IV might regulate *exon IV* expression through impinging on the MAPK-ERK pathway.

3.3. The regulation of BDNF-induced *bdnf* *exon IV* expression by CaM KII/IV is independent of MAPK-ERK pathway

To examine whether BDNF-induced *exon IV* transcription is also regulated by ERK and CaM KII/IV, we pre-treated cortical neurons with U0126 and KN93 before applying BDNF (5ng/ml). Semi-quantitative RT-PCR, as well as real-time PCR, demonstrated that the transcriptional up-regulation of *exon IV* was significantly blocked by U0126 (Fig. 3A). Consistently, BDNF-induced p-ERK activation was also blocked by U0126 (Fig. 3B). We noticed that, while U0126 completely blocked ERK phosphorylation, it only partially (but significantly) inhibited NMDA- and BDNF-mediated *exon IV* transcription. It is conceivable that the partial *exon IV* transcription is supported by other ERK-independent mechanisms. For example, BDNF may regulate chromatin structure, which confers higher binding affinity to CREB in the absence of phosphorylation on Ser133 (Riccio et al. 2006). The partial block of ERK-dependent transcription by U0126 was reported for inflammation-induced *bdnf* transcription (Obata et al. 2003), as well as other immediate early genes (Ravni et al. 2008). In summary, our results implicate that both NMDA- and BDNF-induced *exon IV* transcription require the MAPK-ERK signaling.

We next found that inhibition of CaM KII/IV by KN93 blocked BDNF-induced transcription of exon IV (by semi-quantitative RT-PCR, left and middle panels of Fig. 3C). Consistently, real-time PCR examination confirmed the effects of KN93 on self-mediated transcription of *bdnf* exon IV, whereas the inactive analogue KN92 failed to block the transcriptional up-regulation (right panel of Fig. 3C). In contrast to the NMDA-stimulated neurons, the activation of p-ERK was not affected by KN93 in BDNF-stimulated neurons (Fig. 3D). These data implicated that *bdnf* is differentially regulated in NMDA- and BDNF-stimulated neurons. The requirement of CaM KII/IV activity for self-mediated *bdnf* transcription is independent of MAPK-ERK signaling, and the activation of ERK alone is not sufficient to up-regulate exon IV. We further suggest that CaM KII/IV activity is not required for BDNF-induced ERK activation.

3.4. PI3K and PLC differentially regulate NMDA- and BDNF-induced exon IV transcription

In addition to the ERK/MAPK signaling, PI3K/Akt and PLC- γ are the other two major pathways stimulated by BDNF/TrkB. Because of their roles in synaptic transmission and plasticity, we examined PI3K and PLC function in BDNF-induced *exon IV* transcription. As determined by both semi-quantitative and real-time PCR, it appeared that the up-regulation of *exon IV* was not blocked by PI3K inhibitor LY294002 or PLC inhibitor U73122 (Fig. 4A, B) in BDNF-stimulated neurons. Interestingly, either LY294002 or U73122 significantly blocked NMDA-induced *exon IV* expression (Fig. 4C, D). These data demonstrated that NMDA-induced and self-mediated *exon IV* transcriptions are differentially regulated.

3.5. The activities of PI3K and PLC are required for ERK activation in NMDA-stimulated, but not BDNF-stimulated neurons

Because ERK activity was required for *exon IV* up-regulation in both NMDA- and BDNF-stimulated neurons, we examined whether PI3K and PLC pathways converged on ERK signaling. We found that the NMDA-stimulated activation of p-ERK was blocked by both LY294002 and U73122 (Fig. 5A, B), indicating a cross talk among these pathways. These data also implicate that the regulation of NMDA-stimulated *exon IV* by PI3K and PLC may be mediated through ERK. In contrast to NMDA-stimulated neurons, inhibition of PI3K (by LY294002) and PLC (by U73122) had no effects on BDNF-stimulated ERK phosphorylation (Fig. 5C, D). These results demonstrated that NMDA-, but not BDNF-induced ERK activation depends on PI3K and PLC activity. The distinct cross talk among these signaling pathways may provide the mechanisms for the differential regulation of *bdnf* transcription upon NMDA and self stimulation.

4. Discussion

The activity-dependent up-regulation of *bdnf* transcription is correlated with the induction of LTP and the acquisition phase of memory formation. Because the suppression of BDNF expression blocked memory (Ma et al. 1998), the transcriptional up-regulation of *bdnf* may be functionally relevant. The initiation of activity-dependent *bdnf* transcription is triggered by Ca^{2+} influx through either NMDA receptors or L-type voltage-gated calcium channels (L-VGCC) (Tabuchi et al. 2000; West et al. 2002). A recent finding demonstrated that BDNF stimulates its own transcription through promoter IV in cultured cortical neurons (Yasuda et al. 2007). Intra-hippocampal infusion of BDNF also up-regulated *exon IV* (Wibrand et al. 2006) in the dentate gyrus. It is important to note that the same procedure leads to LTP in the dentate gyrus without high frequency stimulation (Ying et al. 2002). These studies suggested an interesting possibility that the self stimulation might be responsible for the sustained maintenance of *bdnf* expression, which may be important for the maintenance of activity-dependent neuronal changes. In addition to promoting calcium influx, neuronal activity may also stimulate BDNF secretion (Hartmann et al. 2001; Nagappan and Lu 2005; Kolarow et al. 2007), and in turn up-regulate *exon IV* transcription. It is conceivable that the self-stimulation,

following the initial neural stimulation, provides positive feedback mechanisms and is important for the sustained maintenance of BDNF expression involved in certain aspects of neuroplasticity. Because the mechanisms underlying BDNF self-stimulation are largely unknown, we studied the role of the major signaling pathways in *bdnf* transcription with cultured cortical neurons. Here, we confirmed that both NMDA and BDNF stimulated *exon IV* transcription. Our data demonstrated that the NMDA- and BDNF-induced *exon IV* expression is differentially regulated.

Due to the existence of the CRE in the *bdnf* promoter IV the function of the transcription factor CREB was postulated (Tao et al. 1998; Shieh and Ghosh 1999). Indeed, CRE-mediated transcription was observed after the induction of LTP (Impey et al. 1996) and learning-related trainings (such as contextual fear conditioning and passive avoidance) (Impey et al. 1998a). Furthermore, lack of CREB function results in defective LTP and memory formation (Bourtchuladze et al. 1994; Yin et al. 1994). The activation of CREB by phosphorylation at Ser133 recruits another necessary transcription factor CBP (CREB binding protein) to turn on CRE-mediated transcription (Hardingham et al. 1999; Hu et al. 1999). It was hypothesized that Ca^{2+} -stimulated protein kinases are required for the activation of CREB by phosphorylating S133 (Chawla et al. 1998; Shaywitz and Greenberg 1999). Although PKA was identified as the main kinase for CREB initially, ERK may play a major role for the persistent phosphorylation of CREB in neurons (Impey et al. 1998b; Wu et al. 2001). The nuclear-localized CaM KIV only transiently phosphorylates CREB upon membrane depolarization (Bito et al. 1996; Wu et al. 2001), and may be the functional kinase for CBP (Chawla et al. 1998; Impey et al. 2002). CaMK II may have dual effects on CREB, because it may phosphorylate both Ser133 and 142 of CREB. While the phosphorylation on Ser133 activates CREB-mediated transcription, the phosphorylation on Ser142 inhibits the interaction between CBP and CREB (Parker et al. 1998). Here, we demonstrated that ERK activity is necessary for both NMDA- and BDNF-induced *exon IV* transcription.

Although inhibition of CaM KII/IV blocked both NMDA- and BDNF-induced *exon IV* transcription, the underlying mechanisms are different. It appeared that inhibition of CaM KII/IV blocked p-ERK only in NMDA- but not in BDNF-stimulated neurons. Because KN93 blocked BDNF-induced *exon IV* transcription without affecting ERK activation, we suggest that the activation of p-ERK alone is not sufficient for the maintenance phase of BDNF up-regulation.

The role of PI3K in LTP and NMDA-stimulated ERK activation was demonstrated before, suggesting PI3K as an essential up-stream regulator of MAPK signaling (Chandler et al. 2001; Opazo et al. 2003). Here, we confirmed that inhibition of PI3K by LY294002 inhibited NMDA-induced p-ERK. Consistently, the transcription of *exon IV* was blocked by LY294002 in NMDA-stimulated neurons. However, inhibition of PI3K had no effects on either p-ERK or the transcriptional up-regulation of *exon IV*. These results suggest that the cross-talk between PI3K and MAPK may be required for NMDA-dependent, but not BDNF-dependent *exon IV* up-regulation. Interestingly, inhibition of PI3K only impaired the induction, but not the maintenance of HFS-induced LTP (Opazo et al. 2003). It was demonstrated that BDNF-induced LTP in the dentate gyrus depends on MAPK activity (Ying et al. 2002). It would be interesting to examine whether PI3K signaling is required for BDNF-induced LTP.

One interesting finding of this study showed the novel role of PLC in regulating both MAPK-ERK signaling and *exon IV* transcription in NMDA-, but not BDNF-stimulated neurons. PLC hydrolyzes PIP_2 to diacylglycerol (DAG) and inositol-(1,4,5)-triphosphate (IP_3), which promotes calcium release from intracellular storage (Rhee and Choi 1992a, b). Although it was demonstrated that mGluR activation stimulates PLC, a recent report showed that the activation of NMDA receptor is sufficient to trigger the PLC-DAG-PKC pathway in an mGluR-

independent manner (Codazzi et al. 2006). Moreover, PLC activity is required in NMDAR-dependent long-term depression (LTD) (Horne and Dell'Acqua 2007). Our results showed that PLC played an essential role in NMDA-induced *bdnf* transcription. Mechanistically, our data suggest that PLC activity is required for NMDA-induced ERK activation.

Theoretically, PLC-mediated calcium release may regulate ERK phosphorylation upon BDNF stimulation. However, we and others have previously showed that the level of intracellular calcium remained unchanged in BDNF-stimulated cortical neurons (Pizzorusso et al. 2000; Zheng et al. 2008). Because inhibition of PLC had no effects, the BDNF-mediated ERK phosphorylation may only require the basal level of intracellular calcium. Similarly, the basal activity of CaM kinases may gate the BDNF-stimulated exon IV transcription in the absence of PLC-mediated calcium release from internal stores. Although the function of PLC and CaM Kinases was demonstrated for NMDA-dependent plasticity, it would be interesting to examine their roles in BDNF-mediated LTP.

In summary, we used cultured cortical neurons to study the transcriptional control of the BDNF gene, which has been implicated in many aspects of neuronal function. We demonstrated that the regulatory mechanisms are tailored to different neuronal stimuli. Our results suggest that the induction and maintenance phases of *bdnf* transcription may be differentially regulated.

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Fig. 1.

NMDA and BDNF stimulate *bdnf* exon IV expression by activating transcription without affecting mRNA stability. A) NMDA-mediated *exon IV* mRNA up-regulation depends on NMDA receptors. Cortical neurons were stimulated by NMDA with or without NMDAR antagonist APV. B) When overall transcription was blocked by actinomycin (ACD), the level of exon IV-containing mRNA was similar between control and NMDA-treated neurons. C) Self stimulation of *exon IV* expression is achieved by sub-nanomolar BDNF, and depends on the activity of TrkB. D) When overall transcription was blocked by actinomycin (ACD), the level of *exon IV*-containing mRNA was similar between control and NMDA-treated neurons. The mRNA level of *bdnf* and *GAPDH* was determined by semi-quantitative RT-PCR using

gene specific primers. The representative images are shown in the left panels. The level of the housekeeping gene *GAPDH* was used for normalization and quantification (shown in the right panels; n=3 from separate experiments). *, p<0.05 compared to the control level. #, p<0.05 compared to the induction level.

Fig. 2.

Calmodulin-dependent protein kinases II and IV regulate NMDA-induced *bdnf* exon IV expression through MAPK-ERK pathway. A) Inhibition of MEK by U0126 blocked NMDA-induced exon IV up-regulation. B) Inhibition of MEK by U0126 blocked NMDA-induced activation of p-ERK. C) Inhibition of CaM KII/IV by KN93 blocked NMDA-induced exon IV up-regulation. No inhibition was observed for application of its inactive analogue KN92. D) Inhibition of CaM KII/IV by KN93 blocked NMDA-induced activation of p-ERK. The mRNA level of exon IV was determined by semi-quantitative and real time RT-PCR normalizing to GAPDH (optical density or comparative threshold cycles). The activated form of ERK was determined by Western blots using phospho-specific antibodies against p-ERK and normalized to the level of total ERK (T-ERK). The representative images are shown in the left panels. The quantification of mRNA from semi-quantitative RT-PCR are shown in the middle panels and that from real time PCR are shown in the right panels (n=3 from separate

experiments). Representative images for Western blot are shown in the left panels and quantifications are in the right panels. *, $p < 0.05$ compared to the control level. #, $p < 0.05$ compared to the induction level.

Fig. 3.

The regulation of BDNF-induced *BDNF exon IV* expression by CaM KII/IV is independent of MAPK-ERK pathway. A) Inhibition of MEK by U0126 blocked BDNF-induced *exon IV* up-regulation. B) Inhibition of MEK by U0126 blocked BDNF-induced activation of p-ERK. C) Inhibition of CaM KII/IV by KN93 blocked BDNF-induced *exon IV* up-regulation. The inactive form KN92 had no effect. D) Inhibition of CaM KII/IV by KN93 had no effects on BDNF-induced activation of p-ERK. Left panels are representative images. Middle panels show the quantification of semi-quantitative PCR. Right panels show the quantification of real time PCR (A and C) or Western blot (B and D) (n=3 from separate experiments). *, p<0.05 compared to the control level. #, p<0.05 compared to the induction level.

Fig. 4.

PI3K and PLC activity are required for NMDA-induced but not BDNF-induced *bdnf* exon IV expression. Cortical neurons were pre-treated with PI3K inhibitor LY294002 (30uM) (A and C) or PLC inhibitor U73122 (5uM) (B and D) for 30min before BDNF (5ng/ml) (A and B) or NMDA (C and D) application. Left panels are representative images. Middle panels show quantification from semi-quantitative PCR measurements (n=3 from separate experiments). Right panels show quantification from real time PCR measurements. *, p<0.05 compared to the control level. #, p<0.05 compared to the induction level.

Fig. 5. PI3K and PLC activity regulate NMDA-induced, but not BDNF-induced, activation of ERK. Cortical neurons were pre-treated with PI3K inhibitor LY294002 (30uM) (A, C) or PLC inhibitor U73122 (5uM) (B, D) for 30min before NMDA (A, B) or BDNF (C, D) application. The activated form of ERK was determined by antibodies against p-ERK. Left panels are representative images. Right panels show quantification (n=3 from separate experiments). *, $p < 0.05$ compared to the control level. #, $p < 0.05$ compared to the induction level.

Table 1

Primer sequences used in RT-PCR

Gene	Primer sequences
<i>BDNF</i> exon I	Forward 5'-ACTCAAAGGGAACGTGTCTC-3'
	Reverse 5'-GCCTTCATGCAACCGAAGTA-3'
<i>BDNF</i> exon II	Forward 5'-CGGTGTAGGCTGGAATAGAC-3'
	Reverse 5'-GCCTTCATGCAACCGAAGTA-3'
<i>BDNF</i> exon IV	Forward 5'-CTCCGCCATGCAATTCCAC-3'
	Reverse 5'-GCCTTCATGCAACCGAAGTA-3'
<i>BDNF</i> exon VI	Forward 5'-GTGACAACAATGTGACTCCAC-3'
	Reverse 5'-GCCTTCATGCAACCGAAGTA-3'
<i>GAPDH</i>	Forward 5'-TCCATGACAACCTTGGCATTGTGG-3'
	Reverse 5'-GTTGCTGTTGAAGTCGACGAGAC-3'