and functions as the upstream regulator of the BDNF-potentiated miR-132 expression.

The miR-132 was involved in the BDNF-dependent increase in expression of postsynaptic proteins

Previously, we reported that BDNF increases synaptic proteins via the MAPK/ERK1/2 pathway (Matsumoto et al., 2006; Kumamaru et al., 2008). Therefore, to verify the possible involvement of miR-132 in the regulation of pre- and postsynaptic protein expression, the effect of an antisense oligonucleotide to inhibit the miR-132 function was examined. Consistent with our previous studies, BDNF significantly increased postsynaptic proteins (ionotropic glutamate receptors) NR2A, NR2B, and GluR1. Interestingly, these increases were weakened by the antisense transfection (Fig. 4A). As shown, miR-132 antisense partly reduced the BDNF-induced upregulation of glutamate receptor subunits. There may be an alternative mechanism (including regulation by other unknown miRs) that also controls the BDNF-increased glutamate receptors. The level of TUJ1 (class III β-tubulin, a neuronal marker) is shown as a control (Fig. 4A). Levels of presynaptic proteins, including synapsin I, SNAP25, and syntaxin were also checked. In contrast to postsynaptic proteins, no change in the levels of these presynaptic proteins was caused by miR-132 antisense with or without BDNF (Fig. 4B). Quantification of these pre- and postsynaptic proteins was performed (Table 1). Furthermore, the effect of exogenous ds-miR-132 (double-stranded synthesized precursor of mature microRNA) transfection was examined. As shown in Fig. 4C, marked upregulation of NR2A, NR2B, and GluR1 was induced by the transfection of ds-miR-132. These results suggest that miR-132 is involved in the BDNF-mediated upregulation of postsynaptic proteins.

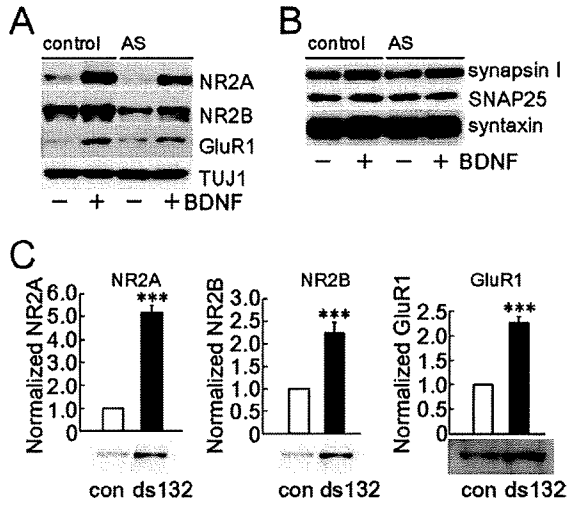

Fig. 4. Antisense RNA transfection to obstruct the function of endogenous miR-132 inhibited the BDNF-increased postsynaptic proteins, and the transfection of ds-miR-132 mimicked the BDNF action. (A) BDNF-increased NR2A, NR2B, and GluR1 were decreased by the miR-132 antisense transfection. TUJ1 (a neuronal marker) is shown as a control. Samples for immunoblotting were collected after BDNF incubation for 24 h. Antisense transfection was carried out 24 h prior to BDNF addition. Quantitative data are shown in Table 1. AS: antisense (B) Presynaptic synapsin I, SNAP25, and syntaxin were also checked. No change in the expression of these presynaptic proteins was caused by miR-132 antisense transfection. Quantitative data are shown in Table 1. (C) Effect of exogenous ds-miR-132 on the postsynaptic proteins. Marked upregulation of NR2A, NR2B, and GluR1 was induced by the transfection of ds-miR-132. ds132: ds-miR-132. After immunoblotting was performed, quantification was carried out. Data represent mean±SD (NR2A; $n=5$, NR2B; $n=4$, GluR1; $n=5$). *** $P<0.001$ (*t*-test).

Table 1. Antisense for miR-132 decreased the BDNF-enhanced expression of postsynaptic proteins, but not presynaptic proteins

	con	BDNF	Antisense	antisense+BDNF
NR2A	0.16±0.13	1.00***	0.11±0.02	0.61±0.15***
NR2B	0.43±0.25	1.00**	0.22±0.13	0.69±0.13
GluR1	0.32±0.09	1.00***	0.38±0.12	0.49±0.12***
Synapsin I	0.84±0.38	1.00	0.54±0.23	1.18±0.24
SNAP25	0.96±0.14	1.00	0.87±0.28	0.93±0.08

Quantification was performed by densitometry after Western blotting. Antisense and BDNF were applied as indicated in the legend for Fig. 4. To quantify Western blot, normalization using response to sole BDNF application was performed. Increase in NR2A and GluR1 expressions by BDNF was significantly attenuated by the antisense for miR-132. The decreasing tendency in the NR2B levels by the antisense was also observed although the statistical significance was not confirmed. In contrast, the levels of presynaptic proteins, including synapsin I and SNAP25 were not changed. Data represent the mean±SD ($n=4$), ** $P<0.01$ (con vs. BDNF), *** $P<0.001$ (BDNF vs. antisense+BDNF). One-way ANOVA followed by Bonferroni's multiple comparison test.

Previously, we showed that DEX inhibits BDNF-increased synaptic proteins via inhibiting ERK1/2 activation in immature hippocampal neurons (Kumamaru et al., 2008). Thus, in the present system, we examined whether there was a decline in the BDNF-stimulated ERK1/2 activation after DEX pretreatment. As expected, DEX suppressed the BDNF-dependent phosphorylation of ERK1/2 (activated ERK1/2 form, pERK1/2) in cultured cortical neurons (a in Fig. 5A). Total ERK1/2 expression was not affected (a in Fig. 5A). Quantification of pERK1/2 levels was carried out (b in Fig. 5A).

As shown in Fig. 3, it is possible that miR-132 expression was regulated via the ERK1/2 pathway. Thus, to further clarify whether ERK1/2 activation is upstream of the BDNF-potentiated miR-132 expression, the effect of miR-132 antisense transfection on the ERK1/2 activation was examined. Surprisingly, ERK1/2 activation by BDNF was diminished by the miR-132 antisense (a, b in Fig. 5B), implying that miR-132 also affects ERK1/2 activation. We examined an effect of miR-132 antisense transfection on the level of TrkB protein because the TrkB receptor is upstream of ERK1/2 activation. As shown, TrkB expression was not influenced by the antisense transfection (a in Fig. 5C). Furthermore, we examined whether BDNF-induced Trk activation is altered by miR-132 antisense. Western blotting using phospho-Trk antibody (Stephens et al., 1994) revealed that an inhibitory effect of miR-132 antisense on Trk activation was not induced (b in Fig. 5C). Lastly, the effect of U0126, an inhibitor of the ERK1/2 pathway, on the upregulation of NR2A, NR2B, and GluR1

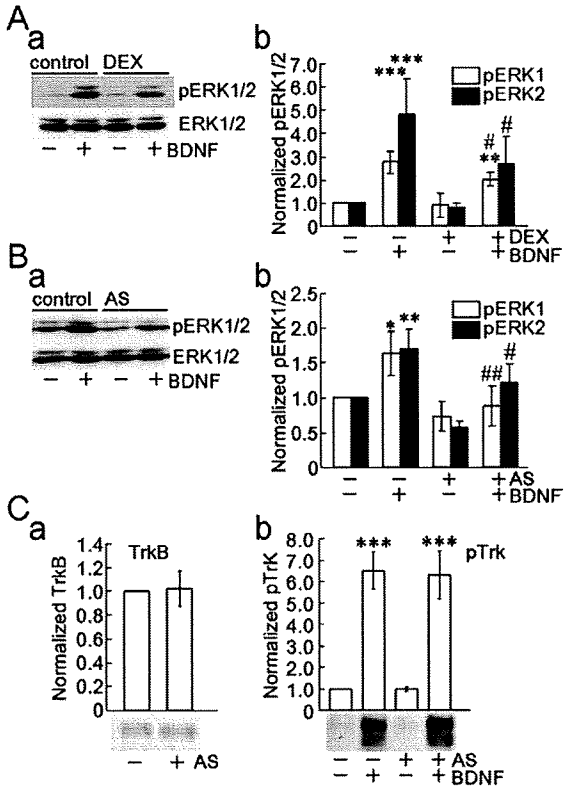

Fig. 5. DEX and miR-132 antisense RNA attenuated the MAPK/ERK1/2 activation stimulated by BDNF. (A) (a) Pretreatment with DEX suppressed the phosphorylation (activation) of ERK1/2 (pERK1/2) stimulated by BDNF. Total ERK1/2 expression was intact. DEX (1.0 μM) was applied at DIV7. Seventy-two hours later, BDNF was added. An additional 24 h of maintenance was conducted before sample collection. (b) Quantification of levels of pERK1/2 was performed. Data represent mean±SD (n=4). *** P<0.001, ** P<0.01 vs. non-treated, # P <0.05 vs. BDNF-treated samples without DEX. One-way ANOVA followed by Bonferroni's multiple comparison test. (B) (a) BDNF-stimulated ERK1/2 activation was diminished by miR-132 antisense. (b) Quantification of levels of pERK1/2. Data represent mean±SD (n=4). ** P<0.01, * P<0.05 vs. non-treated, ## P <0.01, # P <0.05 vs. BDNF-treated samples without antisense. One-way ANOVA followed by Bonferroni's multiple comparison test. (C) (a) Effect of miR-132 antisense transfection on the level of TrkB protein. TrkB expression was not influenced. Data represent mean±SD (n=6). (b) BDNF-induced Trk activation is not altered by miR-132 antisense. Data represent mean±SD (n=8). *** P<0.001 vs. non-treated. One-way ANOVA followed by Bonferroni's multiple comparison test.

caused by ds-miR-132 transfection was examined. U0126 partly but significantly decreased the upregulation of these glutamate receptors, implying that unknown regulation, including involvement of other unknown miRs downstream of ERK1/2 activation, may also contribute to the BDNF-stimulated increase in glutamate receptors (Fig. 6).

DISCUSSION

In the present study, we found that BDNF induced the upregulation of miR-132 through the MAPK/ERK1/2 pathway in cultured cortical neurons. Transfection of antisense RNA to inhibit the miR-132 function diminished the BDNF-dependent increase in the expression of postsynaptic proteins. Transfection of ds-miR-132 upregulated these synaptic proteins in the absence of BDNF. Remarkably, pretreatment with DEX, a synthetic glucocorticoid, decreased BDNF-increased ERK1/2 activation, miR-132 expression, and postsynaptic protein levels.

MiR-132 is critical for BDNF-stimulated dendritic outgrowth (Vo et al., 2005). Morphological change in dendrites might be associated with synaptic connections. Thus, a possible involvement of miR-132 in the maintenance of pre- and postsynaptic protein expressions is an interesting issue. We previously reported that BDNF upregulates synaptic proteins in cultured hippocampal and cortical neurons (Matsumoto et al., 2006; Kumamaru et al., 2008). Thus, in the present study, a possible role of miR-132 in the BDNF-regulated expression of synaptic proteins was examined. BDNF dramatically increased various synaptic proteins, including NR2A, NR2B, GluR1, and synapsin I in cultured cortical neurons. Importantly, the inhibitory effect of miR-132 antisense on the BDNF-increased postsynaptic proteins (especially, NR2A and GluR1) was considerable. On the other hand, presynaptic synapsin I was not influenced by the miR-132 antisense. These results suggest that miR-132 plays a role in postsynaptic protein expression regulated by BDNF, although roles of miR-132 in presynaptic regulation may be further elucidated if neuronal maturity or cell type is altered.

In the present study, upregulation of post-synaptic proteins following the ds-miR-132 transfection occurred. Le et al. reported that endogenous levels of miR-125b are relatively low, and that consequent transfection of the miR-125b duplex brings miR-125b levels up by ~2^7 fold (Le et al., 2009). In their study, ectopic expression of miR-125b downregulates the target protein p53 by ~40%. Notably, the suppression of p53 achieved by the miR-125b duplex was not stronger compared to the p53 siRNA, implying that a higher concentration of ectopic miR is required. In our case, though endogenous miR-132 was also relatively low, the miR-132 level was significantly upregulated by BDNF application. In our preliminary experiment (using a SH-SY5Y neuroblastoma cell line), transfection of ds-miR-132 caused the upregulation of miR-132 expression by ~2^{10} fold. As a result, the strong expression of miR-132 may influence the expression of post-synaptic proteins via suppression of target gene expression. Recently, Wayman reported that the miR132-p250GAP pathway plays a key role in activity-dependent dendrite growth in cultured hippocampal neurons (Wayman et al., 2008). In their system, bicuculline-mediated inhibition of $GABA_A$ inhibitory tone (to increase spontaneous synaptic activity) and transfection of miR-132 increased total dendritic length. Interestingly, in their data, inhibition of miR-132 by 2′-O-methyl RNA antagonists blocked the bicuculline and exogenous miR-132 actions. However, marked decrease of dendritic length was not achieved by solo 2′-O-methyl RNA antagonist application compared with control. In our present study, the significant downregulation of post-synaptic proteins was not induced by the miR-132 antagonist alone. It is possible that the amount of the miR132 above threshold

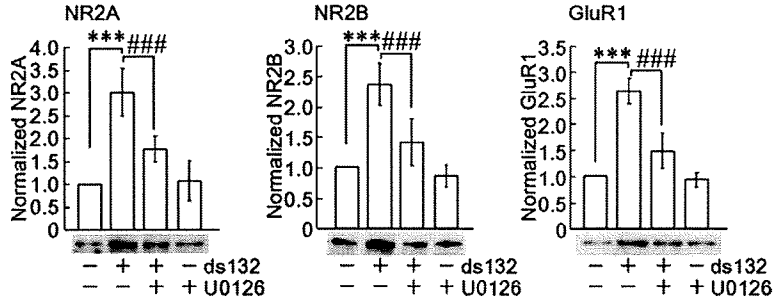

Fig. 6. Effect of U0126 on the upregulation of NR2A, NR2B, and GluR1 caused by ds-miR-132. U0126, an inhibitor of the ERK1/2 pathway, partly but significantly decreased the upregulation of ionotropic glutamate receptors caused by ds-miR-132. Data represent mean±SD (NR2A; $n=8$, NR2B; $n=7$, GluR1; $n=10$). *** $P<0.001$ vs. non-treated. ### $P<0.001$ vs. solo ds-miR-132. One-way ANOVA followed by Bonferroni's multiple comparison test.

may be required for enhanced action of miR-132 in dendritic outgrowth and upregulation of post-synaptic proteins. Moreover, there is a possibility that the miR-132 is important for enhancement of expression of these proteins, but not for basal level maintenance of these proteins.

Chronic stress and glucocorticoid administration causes dendritic atrophy in hippocampal neurons (Woolley et al., 1990; Watanabe et al., 1992; Magariños et al., 1996; Liu et al., 2006). The MAPK/ERK1/2 pathway is important for dendritic formation (Miller and Kaplan, 2003) and we had also confirmed the importance of the activation of ERK1/2 in the neurite outgrowth in developing cortical neurons (Numakawa et al., 2004). Furthermore, we recently found that DEX exhibits an inhibitory effect on BDNF-increased synaptic proteins through preventing ERK1/2 activation in immature hippocampal neurons (Kumamaru et al., 2008). In the present study, we found that BDNF-increased miR-132 was decreased by DEX in mature cortical neurons, and that the BDNF-dependent increase in the miR-132 level was repressed by MAPK/ERK1/2 pathway inhibitors (U0126 and PD98059). In addition to the MAPK/ERK1/2 pathway, the TrkB receptor activates the PI3K pathways, which is critical for cell survival (Patapoutian and Reichardt, 2001; Rodgers and Theibert, 2002; Zheng and Quirion, 2004). In our system, LY294002 (a PI3K inhibitor) had no effect on BDNF-stimulated miR-132 increase. Consistently, neuronal survival was not changed after DEX exposure. Inhibition of the PLC-γ pathway, another downstream signaling of TrkB, had no effect on the miR-132 increase. Therefore, the DEX-dependent prevention of the increase in postsynaptic proteins may be due to diminishing MAPK/ERK1/2 signaling, especially. In our recent study, neurotransmitter release and the activation of PLC-γ stimulated by acute BDNF application after long-term glucocorticoid exposure (24–48 h) were decreased in developing cortical neurons (Numakawa et al., 2009). Collectively, it is possible that glucocorticoids play a variety of functions depending on exposure time to glucocorticoids, neuronal cell types, or their maturity.

The MAPK/ERK1/2 pathway is an important intracellular signaling for the upregulation of miR-132 during BDNF stimulation. With regard to a blocking experiment, the BDNF-dependent miR-132 increase was prevented by U0126 in a dose-dependent manner, suggesting that ERK1/2 activation is upstream of miR-132 upregulation. Unexpectedly, the decrease in the ERK1/2 activation was observed after transfection of the miR-132 antisense, implying that miR-132 has a positive influence on the ERK1/2 activation. Actually, following the miRNA specific target detection algorithm (TargetScan, available at: http://www.targetscan.org/) was used to identify the predicted mRNA targets, we found two putative binding sites of miR-132 within 3′ UTR of ERK1 mRNA (unpublished data). As the first binding site was conserved among vertebrates, we focused on the first binding site for reporter analysis and found that miR-132 decreased reporter activity (unpublished data). However, as shown above, total protein level of ERK1/2 was not affected by DEX and miR-132 antisense in our cultured neurons. In general, it is reasonable that miR-132 antisense blocks the endogenous miR-132 action and increases the levels of both pERK1 and total ERK1, if ERK1 is a target for miR-132. Furthermore, we confirmed that the level of TrkB after miR-132 antisense application was not altered. In addition, BDNF-stimulated Trk activation was not altered by miR-132 antisense. Thus, other molecules including phosphatase targeted by miR-132, that prevents the activation of ERK1/2 or up-stream molecules of ERK1/2, might be a potential target of miR-132 (Fig. 7). Taken together, it is possible that a positive feedback system functions between ERK1/2 signaling and miR-132 for BDNF-dependent upregulation in expression of postsynaptic proteins.

Interestingly, miR-21 regulates the MAPK/ERK signaling pathway in cardiac fibroblasts (Thum et al., 2008). The miR-21 is increased selectively in fibroblasts of the failing heart, augmenting MAPK/ERK activity through inhibition of sprouty homologue 1 (Spry1). Indeed, after the miRNA specific target detection algorithm (TargetScan, available at: http://www.targetscan.org/) was used, a putative binding site of miR-132 within 3′ UTR of Spry1 mRNA was found (data not shown). As Spry1 is a potent inhibitor of the MAPK/ERK pathway (Hanafusa et al., 2002), it is possible that Spry1 may be involved in miR-132-regulated MAPK/ERK signaling, although additional investigation is necessary.

In our cortical cultures, miR-132 expression could be detected, although its level was very low compared with that of miR-9, -124, -128a, or -128b. Interestingly, miR-132

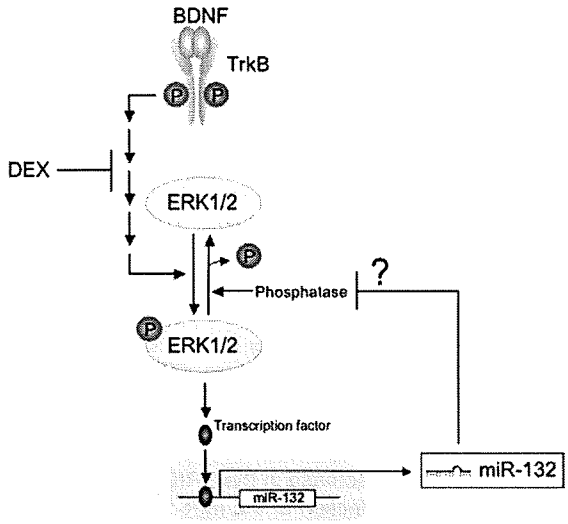

Fig. 7. A positive feedback system functions between ERK1/2 signaling and miR-132. In our system, the MAPK/ERK1/2 pathway is important for the upregulation of miR-132 during BDNF stimulation. Pretreatment with DEX declined BDNF-increased ERK1/2 activation and miR-132 expression. The BDNF-dependent miR-132 increase was attenuated by U0126 and PD98059 (both are ERK1/2 pathway inhibitors), suggesting that activation of ERK1/2 is upstream of miR-132 upregulation. In contrast, miR-132 antisense transfection resulted in decrease in the ERK1/2 activation, implying that miR-132 also has a positive influence on the ERK1/2 pathway. In our cultures, total level of ERK1/2, and expression and activation of Trk were not changed by DEX and miR-132 antisense application. Therefore, unknown phosphatase, that prevents the activations of ERK1/2 or up-stream molecules of ERK1/2, might be targets of miR-132. It is possible that a positive feedback system functions between ERK1/2 signaling and miR-132.

expression was significantly upregulated after BDNF addition. In contrast, the levels of miR-9, -124, -128a and -128b were not affected by BDNF. Thus, it is possible that the function of miR-132 in the cortical system is strictly controlled by BDNF. Computer analysis predicted many candidate genes as targets of miR-132; however, we were unable to identify a direct target involved in the upregulation of ERK1/2 activation or postsynaptic protein expression. In primary human preadipocytes and *in vitro* differentiated adipocytes, miR-132 plays a role in activation of nuclear factor-kappaB (Strum et al., 2009). Very recently, Tai et al. reported that serum- and glucocorticoid-inducible kinase 1, SGK1, directly phosphorylates IKKα at Thr-23 and indirectly activates IKKα at Ser-180, and that the SGK1 phosphorylation of IKKα results in the phosphorylation and activation of nuclear factor-kappaB that consequently upregulates NR2A and NR2B expression (Tai et al., 2009). Klein et al. showed that methyl CpG–binding protein 2 (MeCP2) translation is regulated by miR132 (Klein et al., 2007). Importantly, MeCP2 controls excitatory synaptic strength by regulating the glutamatergic synapse number in hippocampal neurons (Chao et al., 2007). In our cortical cultures, BDNF significantly induced the increase in miR-132 levels and it is possible that miR-132 is important for the BDNF-induced postsynaptic protein expression. The miR-132 is also increased in an activity-dependent manner (Wayman et al., 2008). They showed that neuronal activity inhibited translation of p250GAP (Rho family GTPase-activating protein), a miR-132 target, and siRNA-mediated knockdown of p250GAP mimicked miR-132-induced dendrite growth in hippocampal neurons (Wayman et al., 2008). Taken together, it may be valuable to study whether these miR-132-regulated molecules including nuclear factor-kappaB, MeCP2, and p250GAP are involved in the BDNF-induced postsynaptic protein expression.

Here, we found that DEX exposure negatively regulated BDNF/miR-132 system-mediated glutamate receptor expression in cortical cultures. BDNF is produced and secreted in an activity-dependent manner (Schinder and Poo, 2000; Hartmann et al., 2001; Balkowiec and Katz, 2002). Thus, continuous weak synaptic activity due to the downregulation of glutamate receptors, which is induced by exposure with increased glucocorticoid, may result in the reduction of BDNF protein levels, as observed in depressive disorder (Gervasoni et al., 2005; Karege et al., 2005). Thus, our system, where the upregulation of synaptic proteins occurs through the BDNF/miR-132-dependent signaling, could be used as an *in vitro* model for evaluating novel analogs as antidepressant candidates.

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APPENDIX

Supplementary data

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Review

BDNF function and intracellular signaling in neurons

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Summary. Brain-derived neurotrophic factor (BDNF) and its receptor, TrkB, are broadly expressed in the developing and adult mammalian brain. BDNF/TrkB-stimulated intracellular signaling is critical for neuronal survival, morphogenesis, and plasticity. It is well known that binding of BDNF to TrkB elicits various intracellular signaling pathways, including mitogen-activated protein kinase/extracellular signal-regulated protein kinase (MAPK/ERK), phospholipase Cγ (PLCγ), and phosphoinositide 3-kinase (PI3K) pathways, and that BDNF exerts biological effects on neurons via activation of similar mechanisms. In addition to TrkB, a low-affinity receptor p75 is also involved in neuronal survival and plasticity. BDNF affects neurons positively or negatively through various intracellular signaling pathways triggered by activation of TrkB or p75. From a clinical standpoint, roles of BDNF have been implicated in the pathophysiology of various brain diseases. The stress-induced steroid hormone, glucocorticoid, and BDNF are putatively associated with the pathophysiology of depression. Recent reports, including our studies, demonstrate possible crosstalk between glucocorticoid- and BDNF/TrkB-mediated signaling. Here, we present a broad overview of the current knowledge concerning BDNF action and associated intracellular signaling as it relates to neuronal protection, synaptic function, and morphological change. Furthermore, understanding the secretion and intracellular dynamics of BDNF proteins is critical as the fate of secreted BDNF may contribute to differences in neuronal response.

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Introduction

Neurotrophins, namely nerve growth factor (NGF), BDNF, neurotrophin-3 (NT-3), and NT-4/5, bind to high-affinity Trk receptors, as well as to a common low-affinity p75 receptor. Binding of NGF to TrkA, BDNF and NT-4/5 to TrkB, or of NT-3 to TrkC (weakly to TrkB) leads to activation of various intracellular signaling pathways. It is well known that MAPK/ERK, PLCγ, and PI3K pathways are stimulated after Trk activation (Huang and Reichardt, 2003). Beyond the promotion of cell differentiation, nerve growth, and neuronal survival, the neurotrophin family plays multiple roles in regulating neuronal structure and function in the developing and mature nervous system via activation of various pathways, including the three signaling cascades mentioned above.

BDNF/TrkB-stimulated signaling is necessary for survival and morphogenesis of CNS neurons. Furthermore, it is widely accepted that BDNF plays a critical role in neuronal plasticity. In this article, a broad review of the current literature concerning BDNF-dependent intracellular signaling is described. We focus on the role of BDNF in neuronal survival, synaptic function, and morphological change, in addition to the importance of secretion or intracellular transport of this factor. BDNF is translated as a precursor protein (proBDNF), subsequently proteolytically cleaved to generate a small mature protein (mBDNF). The p75 neurotrophin receptor binds to pro-neurotrophin with high-affinity (Lee et al., 2001) which places great importance on the amount of proBDNF secreted from

neurons. Recent reports suggest that neurons are able to release both proBDNF and mature BDNF (Yang et al., 2009). Interestingly, both low- and high- frequency neuronal activities were shown to increase extracellular levels of proBDNF, though only high-frequency activity induced tissue plasminogen activator secretion. Such secretion of tissue plasminogen activator resulted in extracellular conversion of proBDNF to mBDNF (Nagappan et al., 2009). In this article, we include several reports concerning the involvement of p75 in activity-dependent synaptic plasticity.

As expected, roles of BDNF have been implicated in the pathophysiology of various brain diseases, including depressive disorder. Indeed, many reports suggest that BDNF expression is decreased in several mental disorders, including schizophrenia, bipolar disorder, and major depression (Knable et al., 2004; Gervasoni et al., 2005; Karege et al., 2005). Interestingly, the stress hormone, glucocorticoid, is also putatively associated with the pathophysiology of depression (Watanabe et al., 1992; McEwen, 2005; Kunugi et al., 2006). Thus, it is possible that both BDNF and glucocorticoid are involved in synaptic function and the pathophysiology of depression. As a result, in this article, we provide examples concerning possible crosstalk between BDNF and glucocorticoid actions from some of our current studies.

BDNF signaling in neuronal survival

Trk receptor signaling

BDNF is critical to neuronal survival in the CNS. Moreover, in vivo application of BDNF has been shown to protect a variety of neurons from brain injury (Wu and Pardrige, 1999; Schäbitz et al., 2000). A neuroprotective effect of BDNF was observed when delivered intravenously after the onset of focal cerebral ischemia (Schäbitz et al., 2000). It has also been shown that peripherally administered BDNF plays a neuroprotective role in the brain, after BDNF is reformulated to optimize plasma pharmacokinetics with carboxyl-directed pegylation to enable transport through the blood-brain barrier by coupling to brain transport vectors (Wu and Pardrige, 1999). In vitro evidence, however, demonstrates that BDNF promotes cell survival through activation of TrkB, a high affinity receptor for BDNF. Upon activation, TrkB receptor signaling activates several small G proteins, including Ras, Rap-1, and the Cdc-42-Rac-Rho family, as well as pathways regulated by MAP kinase (MAPK), PI 3-kinase (PI3K) and phospholipase-Cγ (PLCγ) (reviewed by Huang and Reichardt, 2003). Although many intracellular signaling pathways are involved in neuronal survival, the ERK/cAMP-response element binding (CREB) and PI3K/Akt pathways are among the most critical and are the pathways on which BDNF exerts its survival effects. It has been known for many years that activation of ERK, a member of the MAPK family, is involved in BDNF-dependent survival effects (Hetman et al., 1999). In cultured hippocampal neurons, the protective effect of BDNF against glutamate toxicity is mediated by PI3K and the Ras/MAPK signaling cascades (Almeida et al., 2005). Application of norepinephine (NE) increases BDNF and phosphorylated Trk, while this increase is prevented by ERK and PI3K inhibitors in hippocampal neurons (Chen et al., 2007). It is hypothesized that NE-induced BDNF expression follows a cyclic pathway, reminiscent of a positive feedback loop. This is because phosphorylation of the CREB protein was also increased by NE and reduced by MAPK and PI3K inhibitors, and because these inhibitors suppress phosphorylation of TrkB and CREB, respectively. N-Methyl-D-aspartate (NMDA) also promotes neuronal survival against glutamate-mediated excitotoxicity via a BDNF autocrine loop in cultured cerebellar granule cells (Zhu et al., 2005). In their system, NMDA receptor activation caused a concentration- and time-dependent activation of MAPK. This activation was blocked by an NMDA receptor antagonist and was attenuated partially by the tyrosine kinase inhibitor k252a, suggesting that activation of both NMDA and TrkB receptors are required for neuroprotection.

Recently, it has been confirmed that various survival factors, including cyclic adenosine monophosphate (cAMP), PACAP and cell depolarization elicited by high-KCl, are able to induce the activation of ERK in cerebellar granule cells (Obara et al., 2007). The literature addressing the biological effects of ERK on survival, however, demonstrates much controversy. Insulin-like growth factor 1 (IGF-1) and BDNF prevent hippocampal neurons from serum deprivation-induced cell death (Zheng and Quirion, 2004). IGF-1 and BDNF induce this neuroprotection by stimulating activation of the PI3K and MAPK pathways. Interestingly, only the inhibitor of the PI3K pathway was able to block the survival effects elicited by IGF-1 and BDNF, while an inhibitor of the MAPK pathway had no effect. Moreover, in HT22 cells that derive from a murine cell line of hippocampal origin, the pro-apoptotic function of ERK was demonstrated through persistent activation caused by glutamate-induced oxidative toxicity (Stanciu et al., 2000). To expand upon these findings, Rössler et al (2004) showed that the biological endpoint of transient versus sustained activation of ERK was strikingly different. While transient ERK activation by BDNF did not rescue HT22 cells after serum deprivation, the sustained activation of ERK by a conditionally active form of Raf-1 was effective on cell survival. In cultured cortical neurons, exposure to oxidative stress triggers a series of events including over-activation of ERK and intracellular Ca^{2+} accumulation via voltage-gated Ca^{2+} channels and ionotropic glutamate receptors, ultimately resulting in neuronal cell death (Numakawa et al., 2007). Under such oxidative stress, the ERK1/2 signal may work as a death mediator, as the MAPK pathway

inhibitor blocks the oxidative stress-induced ERK1/2 activation, Ca^{2+} overload, and consequent cell death. Comparing the neurotrophic actions of fibroblast growth factor-2 (FGF-2), IGF-1 and BDNF in hippocampal neurons, all three factors promote neuronal survival under serum-free, low-insulin conditions (Johnson-Farley., 2007). Co-treatment with either IGF-1 or BDNF enhanced FGF-2-stimulated Akt and ERK activation, though no enhancement of survival beyond that achieved by solo FGF-2 application was observed with co-treatment. As described, the MAPK/ERK signaling pathway plays a critical role in neuronal survival, while also influencing death signaling. Activation of the MAPK/ERK signaling pathway above threshold may be toxic, while basal activity of this pathway is necessary for maintenance of neuronal survival.

Many reports support a survival function to activation of the PI3K pathway (reviewed by Kaplan and Miller, 2000; Yuan and Yankner, 2000). Akt, a serine/threonine kinase, is a downstream mediator of PI3K activation (Franke et al., 1997), and the PI3K/Akt pathway is critical for neurotrophin-dependent survival in CNS neurons (reviewed by Brunet et al., 2001). Importantly, Almeida et al. (2005) showed that the PI3K/Akt pathway is involved in neuroprotection through BDNF application in cultured hippocampal neurons. In their system, PI3K inhibitors significantly decreased the activation of ERK1/2. Inhibition of MEK (MAPK/ERK kinase), on the other hand, had a minor effect on Akt activation, suggesting that the PI3K pathway is the predominant mechanism by which BDNF acts to stimulate the MAPK pathway in hippocampal neurons. The PI3K/Akt survival pathway is activated after adding ganglioside GM1 to striatal slices (Duchemin et al., 2008). PI3K activity was increased in Trk and Gab1 immunoprecipitates, and co-immunoprecipitation experiments demonstrated the association of Trk and Gab1 after GM1 application. GM1, however, did not transactivate Trk, having no effect on the subsequent release of endogenous neurotrophins (NGF, NT-3, and BDNF). This suggests that GM1 stimulates activation of PI3K, in part, through Trk and Gab1.

Endocytosis of Trk receptors is critical for neurotrophin-dependent biological functions (Grimes et al., 1996). Interestingly, blocking endocytosis in cultured CNS neurons inhibits BDNF-induced activation of Akt, but not activation of ERK (Zheng et al., 2008). As expected, such endocytosis-dependent activation of Akt is important for survival-promoting effects of BDNF. Overall, in contrast to activation of the MAPK/ERK pathway, there is accumulating evidence that activation of the PI3K/Akt pathway acts as the predominant survival-promoting signal.

BDNF has been implicated in the pathophysiology of neuroprotection and thought of as a potential treatment for neurodegeneration. Cystamine (CYS), an anti-oxidant and anti-apoptotic compound, increased BDNF protein levels in frontal cortex tissue seven days after treatment (Pillai et al., 2008). CYS protects cortical neurons through a mechanism involving TrkB receptor activation, and a signaling pathway involving PI3K and MAPK/ERK. Leptin, an adipose hormone, protects against delayed neuronal cell death in hippocampal CA1 following transient global cerebral ischemia (Zhang and Chen, 2008). Leptin increases expression of BDNF and the phosphorylation of Akt and ERK1/2 in the CA1 region after ischemia. Furthermore, the potential therapeutic value of BDNF to Huntington's disease (HD) has been a topic of recent importance. Gharami et al (2008) employed over-expression of BDNF in the forebrain of R6/1 mice which express a fragment of mutant huntingtin with a 116-glutamine tract. Indeed, the BDNF overexpression increased TrkB/Akt signaling activity in the striatum, ameliorated motor dysfunction, and reversed brain weight loss in R6/1 mice.

Trk receptors can also stimulate the PLCγ pathway, which is known to activate the transient receptor potential cation channel (TRPC) (reviewed by Clapham, 2003). Recently, Jia et al., (2007) showed that two members of the TRPC subfamily, TRPC3 and 6, prevented cerebellar granule neurons (CGN) from cell death in cultures and supported survival of CGN in rat brain. It is well known that activation of PLCγ is stimulated by BDNF hydrolyses of PtdIns (4, 5)P2 to generate inositol tris-phosphate (IP_3) and diacylglycerol, where IP_3 is required for intracellular Ca^{2+} channel activation (reviewed by Huang and Reichardt, 2003). BDNF induced an intracellular Ca^{2+} increase via IP_3-sensitive Ca^{2+} channels in cultured cortical neurons (Numakawa et al., 2002a,b). Notably, increases in intracellular Ca^{2+} consequently regulate BDNF expression (Shieh et al., 1998; Tao et al., 1998). In addition, BDNF is produced and released in an activity-dependent manner (Hartmann et al., 2001; Balkowiec et al., 2002). Taken together, the BDNF-stimulated PLCγ/TRPC/Ca^{2+} signaling may induce an increase in BDNF protein that serves to exert long-lasting effects on CNS neurons through the above positive feed-forward loops.

p75-dependent signals

Interestingly, a low-affinity receptor p75 binds to pro-neurotrophin with high-affinity (Lee et al., 2001). p75 is the first neurotrophin receptor to be discovered in the receptor family that includes Fas and tumor necrosis factor receptors (Chao, 1994). p75 binds all neurotrophins (including NGF, BDNF, NT-4/5, and NT-3), and transmits both positive and negative intracellular signals (reviewed by Kaplan and Miller, 2000). In early studies, the p75 receptor was found to be necessary for amplifying Trk-mediated biological effects (Hempstead et al., 1991; Davies et al., 1993; Barker and Shooter, 1994). Furthermore, transcription factor nuclear factor B, c-Jun amino-terminal kinase, and ceramide generation

were reported as p75-dependent signals (Dobrowsky et al., 1994; Carter et al., 1996; Casaccia-Bonnefil et al., 1996). Authors went on to further classify roles of c-Jun amino-terminal kinase, mixed lineage kinase, and p53 in the neurotrophin-dependent neuronal cell death paradigm (Aloyz et al., 1998; Bamji et al., 1998; Friedman, 2000; Xu et al., 2001).

As described above, BDNF (mature BDNF) promotes neuronal survival via TrkB, however, mature BDNF is initially synthesized as a precursor, proBDNF. In contrast to mature proteins, proforms preferentially activate p75 to mediate neuronal cell death. Therefore, the amount of proBDNF secreted from neurons is a critical issue. Recently, Yang et al (2009) reported that neurons are able to release both proBDNF and mature BDNF. The highest levels of proBDNF were observed perinatally and declined with age, though the proform was still detectable in adulthood. In cultured hippocampal neurons, both low- and high- frequency neuronal activities increased extracellular levels of proBDNF (Nagappan et al., 2009). Surprisingly, only high-frequency activity induced tissue plasminogen activator secretion, resulting in extracellular conversion of proBDNF to matureBDNF. Boutilier et al. (2008) showed that inhibiting endocytosis reduced TrkA (receptor for NGF) activation stimulated by proNGF, but did not have the same effect on mature NGF in PC12 cells. In their system, endocytosis and cleavage appear to be essential for proNGF-induced TrkA activation. They also demonstrate that proBDNF induces activation of TrkB in cerebellar granule neurons and that proBDNF cleavage by furin and metalloproteases facilitates this effect.

BDNF signaling in synaptic function

BDNF, synaptic plasticity, and neurotransmitter release

Besides having long-term effects, including neuroprotection in the CNS, neurotrophins play a fundamental role in neuronal plasticity in the short-term (reviewed by Thoenen, 1995). For many years, it has been recognized that BDNF is essential for neuronal transmission and activity-dependent neuronal plasticity (Lessmann et al., 1994; Kang and Schuman, 1995; Levine et al., 1995; Berninger and Poo, 1996; Korte et al., 1996; Patterson et al., 1996; Li et al., 1998). Long-term potentiation (LTP) is the most studied form of synaptic plasticity. As expected, it has been widely accepted that BDNF is involved in the underlying mechanisms of LTP induction and maintenance (reviewed by Lu et al., 2008). Markedly, in addition to TrkB, p75 is also involved in activity-dependent synaptic plasticity (Rösch et al., 2005). Although LTP was unaffected in hippocampal slices prepared from p75-deficient mice, hippocampal long-term depression (LTD) was impaired. Furthermore, in the hippocampus of p75-deficient mice, the expression levels of two (RS)-alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunits, GluR2 and GluR3, were significantly altered. Moreover, proBDNF facilitates hippocampal LTD via activating p75 (Woo et al., 2005). In their system, deletion of p75 in mice selectively impaired the NMDA receptor-dependent LTD, although other forms of synaptic plasticity were not affected.

As the first step of synaptic plasticity, the release of neurotransmitters is an essential component. BDNF plays a critical role in synaptic function, especially of the glutamatergic synapses (reviewed by Carvalho et al., 2008). BDNF was reported to enhance the depolarization-evoked release of glutamate from isolated cortical and hippocampal nerve terminals (Sala et al., 1998; Jovanovic et al., 2000; Pereira et al., 2006). Jovanovic et al. (2000) showed that BDNF increased MAPK-dependent synapsin I phosphorylation and acutely facilitated evoked glutamate release. They found that a MAPK inhibitor markedly decreased synapsin I phosphorylation and concomitantly reduced neurotransmitter release. Activation of the MAPK/ERK pathway is involved in the BDNF-dependent enhancement of glutamate release evoked by depolarization in cultured cortical neurons (Matsumoto et al., 2001, 2006). PLCγ also contributes to the effects of BDNF by inducing glutamate release in cultured cerebellar and cortical neurons (Numakawa et al., 2001, 2002a). In both neuronal cultures, activation of PLCγ induced release of Ca^{2+} through intracellular Ca^{2+} storage reserves, while the BDNF-induced glutamate release depended on the intracellular Ca^{2+} increase via IP_3-sensitive Ca^{2+} channels (IP_3 receptors). Yang et al. (2001) showed that NT3-induced potentiation of synaptic transmission at neuromuscular synapses was blocked by a PI3K inhibitor, but not by a MAPK inhibitor. Neither stimulation of Ca^{2+} release from intracellular stores by IP_3, nor constitutively active PI3K in presynaptic terminals alone enhanced transmission. Application of IP_3 in neurons expressing constitutively active PI3K elicits a significant synaptic potentiation, suggesting that concomitant activation of PI3K and IP_3 receptors (downstream of PLCγ) is both necessary and sufficient. Interestingly, GIPC1 protein (GAIP (G Alpha Interacting Protein)-interacting protein, C terminus) binds to myosin VI (Myo6) as well as Trk receptors (Yano et al., 2006). Myosin VI (Myo6) and a Myo6-binding protein, GIPC1, were necessary for BDNF/TrkB-mediated facilitation of hippocampal LTP and BDNF-mediated enhancement of glutamate release from presynaptic terminals. Paredes et al. (2007) found that BDNF and NGF, when administered into the hippocampus (using in vivo techniques), evoked dopamine and glutamate release. The BDNF-induced neurotransmitter release was partially blocked by an antagonist for Trks, indicating that BDNF functions via Trk receptors to induce neurotransmitter release. In primary mesencephalic neuronal cultures, acute activation of TrkB by BDNF significantly increased

dopamine uptake (Hoover et al., 2007). The effect of BDNF on dopamine transporter activity was dependent on both the MAPK and PI3K pathways. In contrast, BDNF injection (either intracerebroventricularly or directly into the CA3 region of the hippocampus) decreased the signal amplitude and clearance rate produced by exogenously applied 5-HT (serotonin) (Benmansour et al., 2008). BDNF may play many roles in the control of neurotransmitter levels in synaptic sites. The fusion of synaptic vesicles to the plasma membrane at the nerve terminal is an essential process of exocytotic release of neurotransmitter. During exocytosis, synaptic vesicle-associated synaptic proteins (SV-proteins; synapsin I, synaptotagmin, synaptobrevin, synaptophysin, etc.) and plasma membrane-associated synaptic proteins (PM-proteins; syntaxin and SNAP25, etc.) are involved (reviewed by Südhof, 1995). BDNF application to a hippocampal slice culture obtained from P7 rats for 48 h augmented the expressions of SV-proteins (synaptotagmin, synaptophysin, and synaptobrevin, but not synapsin I), but not of PM-proteins (syntaxin and SNAP25) (Tartaglia et al., 2001). The longer stimulation with BDNF to cultured cortical neurons (prepared from E17 rats) for five days (Takei et al., 1997) or to hippocampal neurons (from E18 rats) for 7-10 days (Yamada et al., 2002) induced the increase in both SV- and PM-proteins. With regards to intracellular signaling, BDNF increases the levels of SV-proteins (synapsin I, synaptotagmin, and synaptophysin) via the PLCγ and MAPK pathways, although levels of PM-proteins (syntaxin and SNAP25) were not changed (Matsumoto et al., 2006). Interestingly, neuronal activity was necessary for the up-regulation of synapsin I, synaptotagmin, and synaptophysin expression, and PLCγ inhibitor attenuated BDNF-stimulated long-lasting MAPK/ERK activation. As BDNF potentiates glutamatergic transmission through the PLCγ pathway (Numakawa et al., 2002a), it is possible that PLCγ-mediated neuronal activity may sustain MAPK/ERK activation, resulting in an increased expression of synaptic proteins. In developing hippocampal neurons, the involvement of the MAPK/ERK pathway in BDNF-dependent upregulation of synapsin I and synaptotagmin was also confirmed (Kumamaru et al., 2008).

BDNF and ionotropic glutamate receptors

It has been widely accepted that functional alterations of ionotropic glutamate receptors, including AMPA and NMDA receptors, are involved in synaptic plasticity. Phosphorylation of AMPA receptors, or their cycling into and out of synaptic sites, is critical for mediating excitatory neurotransmission and plasticity (Carroll et al., 1999; Lissin et al., 1999; Beattie et al., 2000; Wu et al., 2004). The function of postsynaptic glutamate receptors is also regulated by BDNF. Ca^{2+} transients evoked by BDNF induce translocation of GluR1, but not NMDA receptors, to the postsynaptic membrane in cultured cortical neurons (Nakata and Nakamura, 2007). The GluR1 trafficking regulated by BDNF occurs via IP_3 receptor- and TRPC-dependent Ca^{2+} signaling. Local regulation of mRNA translation may play an important role in synaptic plasticity as well. Schratt et al (2004) found subsets of neuronal mRNAs demonstrating enhanced translation after exposure to BDNF. Many proteins that are known to function at synapses, including Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII), NR1, and the postsynaptic density (PSD) scaffolding protein Homer2 were observed. BDNF regulates the translation of Homer2 locally in the synaptodendritic compartment by activating translational initiation via a mammalian target of the rapamycin (mTOR)-PI3K-dependent pathway.

BDNF acutely up-regulates GluR1, GluR2, and GluR3 subunits in cultured hippocampal neurons (Caldeira et al., 2007a). The increase in GluR1 and GluR2 protein levels was impaired by a Trk inhibitor, and by translation and transcription inhibitors. In the appropriate culture, acute stimulation with BDNF selectively increased the level of GluR1 associated with the plasma membrane. Furthermore, BDNF induced GluR1 phosphorylation on Ser-831 through activation of protein kinase C (PKC) and CaMKII. Interestingly, BDNF enhances expression of GluR1 and GluR2/3 subunits in cortical neurons via the Src-family protein tyrosine kinases (PTKs) (Narisawa-Saito et al., 1999). The increase in AMPA receptor levels was blocked by a Src-family-selective PTK inhibitor. Moreover, cortical cultures from Fyn-knockout mice failed to respond to BDNF, suggesting that the Src-family kinase, Fyn, plays a crucial role in modulating AMPA receptor expression. Recently, it has been revealed that chronic BDNF application increases levels of NR2A, NR2B, and GluR1 through the MAPK/ERK pathway in developing hippocampal cultures (Kumamaru et al., 2008). BDNF increases the channel opening probability of the NMDA receptor. Whole-cell and single-channel recordings from cultured hippocampal neurons reveal that BDNF augmented glutamate-evoked, but not acetylcholine-evoked, currents and increased NMDA receptor opening probability (Levine et al., 1998). Tyrosine phosphorylation of NR1 and NR2B NMDA receptor subunits by BDNF was also reported. BDNF acutely elicits an increase in phosphorylation of the NR1 in hippocampal neurons. This effect occurred in synaptoneurosomes, which contain both pre- and postsynaptic components (Suen et al., 1997). Furthermore, postsynaptic NR2B was phosphorylated at Tyr1472 by BDNF (Alder et al., 2005). Crozier et al. (1999) reported that BDNF enhanced activity of NR2B-containing NMDA receptors in cultured hippocampal neurons. Interestingly, antagonists of PKC and PKA had no effect on the response to BDNF, whereas an antagonist of CaMKII reduced response to BDNF.

In addition to the contribution to the activity and phosphorylation of NMDA receptors, BDNF increases

the expression of NMDA receptor subunits. In cultured hippocampal neurons, BDNF up-regulates the levels of NMDA receptor subunits, including NR1, NR2A and NR2B associated with the plasma membrane (Caldeira et al., 2007b). Acute stimulation with BDNF up-regulates these protein levels by a mechanism sensitive to transcription and translation inhibitors. As shown above, the signaling involved in NMDA receptor regulation by BDNF is not as well characterized as that of AMPA receptors. Recently, it has been found that BDNF-dependent up-regulation of NR2A and NR2B in developing hippocampal neurons was blocked by MAPK/ERK inhibitor, suggesting involvement of the MAPK/ERK pathway (Kumamaru et al., 2008).

Morphological change in neurites by BDNF

Morphological changes in axons or dendrites induced by BDNF have been investigated from a variety of angles. Earlier studies captured the entire picture of these BDNF effects (Cabelli et al., 1995; Cohen-Cory and Fraser, 1995; McAllister et al., 1995), and subsequent studies described the signal mechanism and neurological roles of morphological changes. Thus, in this section, we focused on the influence of BDNF on morphological changes in neuronal axons, dendrites, and spines.

Axon morphology

One of the most notable effects of BDNF on axon morphology would be the induction of axon branching in CNS neurons. A pioneering study conducted by Cohen-Cory and Fraser revealed that injection of BDNF into the optic tectum of *Xenopus laevis* increased the branching and complexity of optic axon terminal arbors during development (Cohen-Cory and Fraser, 1995). In their system, an injection of neutralizing antibodies for BDNF reduced axon arborization and complexity. This evidence suggested that BDNF was involved in morphological changes in the axon. Infusion of BDNF into the primary visual cortex of cats induced disarrangement of ocular dominant column formation because competitive axon development was impaired (Cabelli et al., 1995). This suggests that strict regulation of BDNF expression is critical for proper formation of axon networks, leading to construction of the ocular dominant column.

Axon collateral branching is initiated by the appearance of localized filopodia, slender actin-rich protrusions. Application of NGF- or BDNF-coated beads to the axons of cultured DRG neurons resulted in the formation of axonal filopodia at sites of bead contact (Gallo and Letourneau, 1998), suggesting that both NGF and BDNF induce initiation of axon branching. The investigation of signal mechanisms involved in BDNF's effect on filopodia is less understood than that of NGF. However, given the similarity of both neurotrophins in signal transduction, the filopodial effect of BDNF might be regulated through activation of the TrkB/PI3K pathway.

It has been widely accepted that BDNF promotes axon elongation, such as sensory and motor axons in the limb bud (Tucker et al., 2001) or hippocampal neuron (Yoshimura et al., 2005). However, some studies suggest that this may not have always been the case. Ozdinler and Macklis (2006) indicated that BDNF enhanced branching and arborization, but not axon outgrowth of corticospinal motor neurons. In the corticospinal motor neurons, IGF-1 specifically caused axon elongation. This report suggests that axonal branching, arborization, and outgrowth could be independently regulated by different growth factors in the same axon. BDNF did not always induce axon elongation. It is possible that the difference in intracellular signaling stimulated by BDNF determines the action of BDNF in axonal morphology.

In general, BDNF is believed to exert a strong ability to promote axon complexity through TrkB signaling. However, BDNF acts as an axon pruning factor via p75 signaling. In sympathetic neurons, NGF is used as a target-derived neurotrophin to mediate competitive innervations between the axon and their targets. In contrast to the BDNF action on axon morphology described above, secreted BDNF from neighboring axons of sympathetic neurons activated p75 and pruned the sympathetic neuronal axon during development (Deppmann et al., 2008; Singh et al., 2008). This evidence suggests that BDNF's effect on axon morphology is dependent on the expression levels of TrkB and/or p75 in target neurons.

In immature neurons, axons are generated from neurites (Dotti et al., 1988). Since one immature neuron has several neurites, one axon should be selected and generated from immature neurites for proper development. In addition to axon branching or elongation, BDNF is also involved in this process. BDNF-induced activation of TrkB/PI3K and the subsequent Akt-GSK3beta-CRMP-2 signal directly control this process (Jiang et al., 2005; Yoshimura et al., 2005). CRMP-2 regulates microtubule assembly by binding to tubulin heterodimers, thereby enhancing axon elongation and branching. Notably, inhibition of CRMP-2 activation caused the formation of multiple axon-like neurites. Knockdown of CRMP-2 caused a marked inhibition of BDNF-induced axon specification and led to the reduction of subsequent axon outgrowth and branching.

BDNF is also a well established chemoattractive factor (Song et al., 1997). The attractive turning of growth cones is influenced by BDNF as an extracellular guidance clue, which triggers extracellular Ca^{2+} influx. In this process, stimulation of TRP channels by BDNF occurs through the activation of the TrkB/PLCγ pathway (Li et al., 2005; Wang and Poo, 2005). Interestingly, both IP_3 and DAG produced by the PLCγ pathway activate the TRP channel (reviewed by Clapham, 2003).

Specifically, DAG directly activates TRP family channels, whereas IP_3 activates them by triggering intracellular calcium increase. Furthermore, a recent study demonstrated another mechanism for BDNF, where it was found to stimulate membrane potential shifts toward depolarization, thus inducing the growth-cone turning direction (Nishiyama et al., 2008).

Dendrite morphology

Dendritic growth is critical for the proper development of neuronal circuits. In addition to the effect on axons, BDNF strongly influences dendritic morphology. It was discovered that the regulation of cortical dendritic growth by BDNF requires endogenous electrical activity (McAllister et al., 1996). BDNF was released from dendrites and cell bodies and acted directly on adjacent neurons to induce dendritic branching and focal dendritic growth in a distance-dependent manner (Horch and Katz, 2002). In slices of ferret visual cortex, BDNF was shown to increase the number of the apical or basal dendrites of pyramidal neurons and its branching (McAllister et al., 1995). The effect of BDNF on dendritic morphology has a laminar specificity and is distinct between apical and basal dendrite. This suggests that the BDNF-induced morphological change in the dendrite was dependent on the neuronal populations and type of dendrites. Such a morphological influence was also different between each neurotrophin, suggesting a complex mechanism underlying dendritic development. These BDNF actions are thought to act through TrkB signaling. A study using TrkB knockout mice indicated that TrkB signaling is important for regulation of dendritic branching of cortical pyramidal neurons in vivo (Xu et al., 2000). Interestingly, pivotal roles have been implicated for the PI3K pathway on the effects of total dendritic branch length, dendritic caliber, and the number of dendrites in cooperation with MAPK/ERK signaling (Dijkhuizen and Ghosh, 2005; Kumar et al., 2005).

Spine morphology

BDNF controls the neuronal network through the rearrangement of synaptic connections and the regulation of spine morphology directly associated with BDNF-dependent synapse modulations (reviewed by Poo, 2001). The use of a genetically-modified mouse indicate the role of BDNF on synapse number. In particular, the number of synapses was increased in BDNF transgenic mice, whereas synapses were decreased in BDNF knockout mice (Causing et al., 1997). BDNF regulates the morphologies of both glutamatergic and GABAergic synapses. Inhibitory GABAergic synapses do not appear to have a dendritic spine structure and BDNF's action on GABAergic synapses are not well understood. Npas4 (Neuronal Pas Domain Protein-4), a transcription factor, was suggested to play a role in the development of inhibitory synapses (Lin et al., 2008). BDNF mediates a portion of the effect of Npas4 on inhibitory synapse development. In contrast to GABAergic synapses, the formation of glutamatergic synapses was associated with structural changes in dendritic spines. In recent years, BDNF-induced morphological changes in dendritic spines were thought to contribute to synaptic rearrangement in neuronal plasticity. Tyler and Pozzo-Miller (2003) investigated the effects of BDNF on spine morphology by classifying them according to categories established by Peters and colleagues (Peters and Kaiserman-Abramof, 1970). Spine morphology was classified into three groups: stubby (type-I), mushroom (type-II), and thin (type-III) types. They found that long-term BDNF treatment increased spine density in apical dendrites of CA1 pyramidal neurons. They also discovered that BDNF increased both the total spine density and the proportion of stubby spines (type-I), which are thought to promote coordinated and widespread Ca^{2+} transients in dendritic and adjacent spines (Tyler and Pozzo-Miller, 2003). In addition, they found a reduction in the proportion of thin spines (type-III), which was thought to isolate Ca^{2+} transients from the parent dendrite and other spines. These results suggest that BDNF controls calcium signaling and regulates local neuronal communication among hippocampal excitatory synapses by promoting the morphological change of spines. Short-term BDNF exposure also induced enlargement of dendritic spines in glutamatergic synapses (Tanaka et al., 2008). It was suggested that such a morphological change in neuronal spines functions as a structural tag for selective trapping of proteins that may play a role in the long-term spine-head enlargement.

BDNF-stimulated changes in spine morphology depended on the activation of TrkB and the increased level of intracellular cAMP, which was presumably induced by monoamine neurotransmitters (Ji et al., 2005). The cAMP enhanced TrkB phosphorylation induced its translocation to the spine, suggesting an involvement of cAMP in full activation of the TrkB translocation. Ji et al. described it as the "gating" effect of cAMP. Following activation of TrkB, activation of PI3K/Akt signaling seems to mainly mediate spine morphology (Kumar et al., 2005). Furthermore, translation of miRNA, miR-134, in dendrites was also involved in morphological change induced by BDNF (Schratt et al., 2006). The miR-134 negatively regulates the size of postsynaptic sites through inhibiting the translation of an mRNA encoding a protein kinase, Limk1, which controls spine development through the regulation of actin filament dynamics by inhibition of ADF (actin-depolymerizing factor)/cofilin.

In summary, BDNF largely affects the overall morphology of neurons through the activation of PI3K, though growth cone turning has been shown to depend on PLCγ signaling. Given that structures of axons, dendrites and spines are regulated via intrinsically

different mechanisms, each specific signaling paradigm would be included on the list of local biological effects elicited by BDNF. To further investigate BDNF-dependent morphological change in neurites, more local signal molecules should be unveiled.

Cholesterol metabolism by BDNF

As described above, BDNF exerts its biological effects on neuronal cells via activating several intracellular signaling cascades. Interestingly, a couple of studies indicate the effect of BDNF on neuronal metabolites. One study revealed BDNF's role in neuronal energy availability (Burkhalter et al., 2003), and another report from our laboratory suggested the important function in cholesterol metabolism. In this section, potential roles of BDNF-induced cholesterol biosynthesis and its association with neuronal function are demonstrated.

Cholesterol and presynaptic function

Cholesterol is an important membrane component, a precursor compound of steroids and other derivatives, and an intracellular signaling molecule. Since cholesterol is such an indispensable molecule, abnormal cholesterol metabolism consequently causes pathogenesis in many tissues (reviewed by Dietschy and Turely, 2001). Although large amounts of brain cholesterol are present in glial cells, biochemical studies on synaptosomes and freeze-fracture electron microscopy indicate the presence of cholesterol in presynaptic terminals (reviewed by Pfrieger, 2003). Several lines of evidence suggest that cholesterol is an essential element of the biogenesis and transport of synaptic vesicles (SV). A study that identified synaptophysin as a major cholesterol-binding protein showed that the regulatory mechanism of SV availability depended on the amount of cholesterol (Thiele et al., 2000). It is possible that the cholesterol-dependent interactions of SV proteins, including the SNARE complex, may affect the process of exocytosis at presynaptic sites. Furthermore, cholesterol was accepted as one of the major components of lipid rafts. Neuronal lipid rafts from brain tissue, as recognized by their detergent-resistant membrane fractions, are enriched by a subset of synaptic proteins (Chamberlain et al., 2001; Lang et al., 2001; Hering et al., 2003). This suggests that the molecular interaction of synaptic proteins with cholesterol might determine their localization, and that a change in cholesterol levels affects presynaptic functions.

Neurotrophin and cholesterol biosynthesis

Mauch et al. (2001) demonstrated the importance of glia-derived cholesterol in synaptic development. Cholesterol enhanced synaptic efficacy, although cholesterol itself demonstrated no effect on synaptic number (Christopherson et al., 2005). It has been shown that a blockade of cholesterol biosynthesis affects the dendritic and axonal development in cortical and hippocampal neurons (Ko et al., 2005). Our study also indicates that cultured cortical and hippocampal neurons actively synthesize cholesterol (Suzuki et al., 2007). It was determined that BDNF enhances cholesterol biosynthesis through the activation of TrkB. Interestingly, the BDNF-mediated increase in cholesterol occurred in rafts with an increase in presynaptic proteins, suggesting the possibility that BDNF induces presynaptic development via controlling the amount of presynaptic cholesterol. Indeed, an electrophysiological experiment revealed that BDNF-dependent cholesterol biosynthesis plays a role in the maturation of a readily releasable pool of synaptic vesicles, suggesting that BDNF-dependent cholesterol biosynthesis is necessary for synapse development. In contrast, a recent study found lower levels of cholesterol biosynthesis in retinal ganglion cell (RGC) neurons compared to glial cells (Nieweg et al., 2009). They concluded that these neurons could not produce cholesterol efficiently and that they depended on an external source of lipids. This contradictory result may suggest that the action of cholesterol biosynthesis is largely dependent on varying cell types. TrkB signaling via cholesterol-rich lipid rafts is important for BDNF function, such as synaptic modulation, calcium signaling, dendritic growth, chemotropic guidance of growth cones, and endocytosis of TrkB (Guirland et al., 2004; Suzuki et al., 2004; Pereira and Chao, 2007; Takemoto-Kimura et al., 2007). However, as a recent study indicated an enhanced signaling of TrkB by cholesterol loss (Martin et al., 2008), further studies are necessary to unveil the relationship between cellular cholesterol and TrkB signaling in detail.

A p75-dependent cholesterol mechanism for biosynthesis was also reported. In a PC12 cell, Yan et al. (2005) first demonstrated that cholesterol biosynthesis plays a role in pro-apoptotic action via p75, although it was not clear whether the cholesterol biosynthesis via p75 occurred in neurons. Recent evidence supports that cholesterol biosynthetic enzymes (3-hydroxy-3-methylglutaryl-coenzyme A reductase and 7-dehydrocholesterol reductase) are co-expressed in p75-positive neurons throughout the adult murine brain (Korade et al., 2007), indicating a possibility that neurotrophin receptor interacting factor (NRIF) is involved in the p75-dependent cholesterol biosynthesis (Korade et al., 2008).

In addition to cholesterol biosynthesis, BDNF influences neuronal energy and amino acid homeostasis. During neuronal development, BDNF increases glucose utilization with the expression of neuronal glucose transporter GLUT3 (Burkhalter et al., 2003). The energy produced from glucose was used to transport extracellular amino acids into the cell to prepare the de

novo protein synthesis induced by BDNF. This energy from glucose would also be used to synthesize cholesterol.

BDNF and mental illness

As mentioned above, BDNF is a critical neurotrophin for the survival and morphogenesis of neurons, playing important roles in the maintainance and plasticity of the neural circuit. Thus, it is possible that BDNF has an additional influence on the human mind and consciousness. In fact, many reports suggest that BDNF expression is decreased in several mental disorders, including schizophrenia, bipolar disorder, and major depression (Knable et al., 2004; Gervasoni et al., 2005; Karege et al., 2005). Patients with these disorders exhibit reduced volume of various brain regions in addition to neuronal atrophy. For example, a lower volume of the hippocampus and amygdala is observed in patients with major depression (Campbell et al., 2004). A reduction of prefrontal cortex volume in schizophrenic patients is reported to be related to the genotype of the BDNF gene (Varnäs et al., 2008). The met polymorphism at codon 66 of the BDNF gene, compared with that of the Val polymorphism, decreases BDNF trafficking (Egan et al., 2003; Chen et al., 2004). In addition, the Val/Met polymorphism correlates with a lower hippocampal volume, which has been implicated in increasing susceptibility to bipolar disorder (Chepenik et al., 2009). Moreover, alterations of brain volume and BDNF genotype are associated with declined working memory and cognitive dysfunction in the disorders listed above (Ho et al., 2006; Gatt et al., 2009). Possible involvement of BDNF via antidepressant mechanisms has been investigated by using animal experiments; chronic treatment of rats with antidepressants increases mRNA levels of BDNF in the hippocampus (Martínez-Turrillas et al., 2005; Castrén et al., 2007). In cultured cortical neurons, the antidepressant, imipramine, potentiates the release of glutamate elicited by acute BDNF application (Yagasaki et al., 2006). Taking all these findings together, BDNF may play a critical role in the pathophysiology of mental disorders.

Stress and BDNF

In addition to genetic predisposition, environmental and life events are closely related to the onset of mental illness (de Kloet et al., 2005; Gatt et al., 2009). Under stressful conditions, glucocorticoid (cortisol in humans, corticosterone in rodents) levels in serum are increased via activation of the HPA-axis (Hypothalamic-Pituitary-Adrenal axis). In general, this adrenal hormone is essential for energy production to cope with stressful situations in addition to suppressing the immune system (Franchimont, 2004). Interestingly, growing evidence suggests that glucocorticoid has an effect on neuronal function. Acutely elevated or low doses of glucocorticoid demonstrate trophic and protective effects on neurons (Gould et al., 1990). For instance, a moderate level of glucocorticoid is required for the cognitive function of rats, which is governed in the prefrontal cortex (Mizoguchi et al., 2004). Neuronal spine generation was increased by glucocorticoid treatment given within an hour in CA1 pyramidal neurons of rat hippocampal slice cultures, suggesting that acute glucocorticoid increases synaptic plasticity (Komatsuzaki et al., 2005). Enhanced excitability of the hippocampal-amygdala system by glucocorticoid is suggested to be important for consolidating the memory of adverse experiences and arousing situations (de Kloet et al., 2005). However, when excessive stress is prolonged, the stress hormone level is no longer under tight control by the HPA-axis. In normal individuals, the activation of the HPA-axis is terminated within a few hours by the negative feedback effect of glucocorticoid. The lost regulation of the HPA-axis may result in the sustained elevation of glucocorticoid, and this abnormally elevated hormone may then damage CNS neurons as well as the HPA-axis (Watanabe et al., 1992; McEwen, 2005). Indeed, abnormally prolonged activation of the HPA-axis is observed in depressive patients (Kunugi et al., 2006). How does the BDNF level respond to such stressful conditions? Many reports show a significant decrease of BDNF in the brains of animals under stress (Schaaf et al., 2000; Duman and Monteggia, 2006). Glucocorticoid administration also decreases BDNF expression in the hippocampus (Hansson et al., 2003; Smith et al., 1995). Therefore, the stress-induced reduction in BDNF expression may be a part of the pathophysiology of mental disorders. However, the involvement of glucocorticoids in BDNF-activated signaling pathways needs to be studied further.

Stress hormones interfere in BDNF-stimulated intracellular signaling

We recently examined the effect of dexamethasone exposure (DEX, a synthetic glucocorticoid, selective ligand for glucocorticoid receptor, GR) on immature cultured hippocampal neurons (Kumamaru et al., 2008). The hippocampus is a critical region for regulating HPA-axis activity. As we previously reported, DEX pretreatment inhibits the BDNF-dependent dendrite outgrowth in developing neurons (Fig. 1A). Accordingly, BDNF-enriched synaptic proteins, including NR2A (Fig. 1B), NR2B, GluR1, and synapsin I (Fig. 1B), were suppressed by DEX, and the inhibitory action of DEX influenced neuronal function even after the neurons had matured (Kumamaru et al., 2008). The inhibitory action of DEX on the BDNF effect may occur via inhibition of the BDNF-activated MAPK/ERK1/2 pathway (Fig. 1C), which is an important pathway for synaptic maturation and dendritic outgrowth (Numakawa et al., 2004; Matsumoto et al., 2006; Kumamaru et al., 2008). The negative effect of DEX on

the MAPK/ERK1/2 pathway was reported in the analysis of another growth factor - PDGF. PDGF (platelet-derived growth factor) induced the activation of MAPK/ERK1/2, while pretreatment with DEX inhibited such PDGF effects (Obradovic et al., 2006). Reduced activation of ERK1/2 was observed in the hippocampus of mice after they were chronically administered corticosterone in vivo (Gourley et al., 2008). In contrast, it is also reported that stress acutely activates the MAPK/ERK1/2 pathway (Yang et al., 2004). It is possible that cells, including neurons, have negative feedback systems in place to stop the activated signaling within a certain time-frame. Sustained elevation of glucocorticoid may exhaust the ERK1/2 activation by affecting any number of unknown phosphatases. Recent evidence demonstrates that many phosphatases participate in the dephosphorylation of the phosphorylated-ERK1/2 (Keyse, 2000). Indeed, it is reported that glucocorticoid induces the expression of MAPK phosphatase-1 (MKP-1), resulting in a decrease in the phosphorylation of MAPK/ERK1/2 in the mast cell line (Kassel et al., 2001).

Long-lasting changes of neural function caused by glucocorticoids may be involved in the pathophysiology of mental disorders caused by early life stress as reviewed by Cirulli et al., (2009). Prenatal and neonatal rats that received glucocorticoid treatment exhibited an increased brain ventricular volume in addition to decreased spatial memory (Kamphuis et al., 2003; He et al., 2004). The infants of rats repeatedly separated from their mothers demonstrated decreased neurogenesis in the hippocampus, reduced working memory, and exhibited anxiety-like behavior after maturing (Huot et al., 2002; Mirescu et al., 2004). Maternal-separation is also suggested to influence the development of the HPA-axis (Liu et al., 1997). Epigenetic modulation of GR expression is suggested to be involved in the maturation of the HPA-axis (Weaver et al., 2004). In frequently nursed rat infants, GR expression was increased in the hippocampus via reducing the methylation of the promoter region of the glucocorticoid receptor gene. In contrast, GR expression was kept at a low level in 'rarely handled' infants, resulting in elevated activity of the HPA-axis, lowered synaptogenesis in the hippocampus, and poor spatial memory after reaching adulthood (Liu et al. 2000). These findings suggest that an adequate level of GR expression in the CNS is needed for normal regulation of the HPA-axis. Maternal separation during the neonatal period was shown to influence BDNF expression levels in the hippocampus after maturation (Roceri et al., 2002; Cirulli et al., 2009). Prevention of trophic signaling of BDNF during

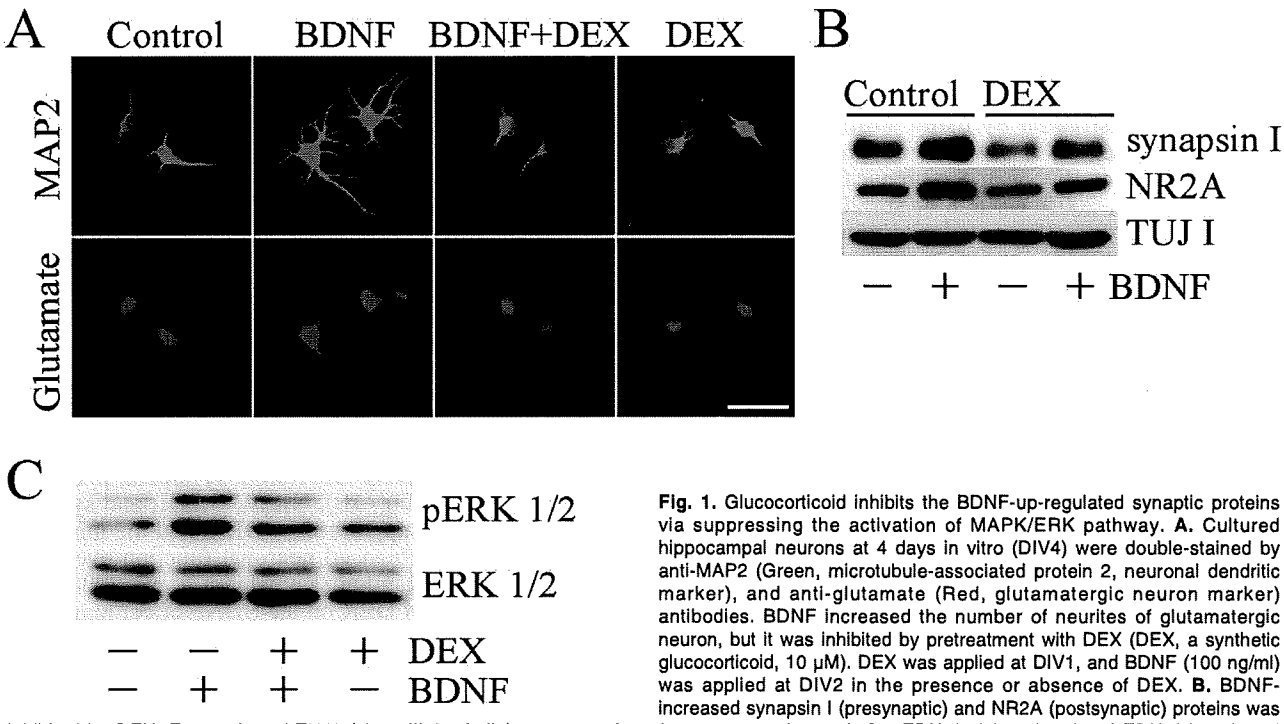

Fig. 1. Glucocorticoid inhibits the BDNF-up-regulated synaptic proteins via suppressing the activation of MAPK/ERK pathway. **A.** Cultured hippocampal neurons at 4 days in vitro (DIV4) were double-stained by anti-MAP2 (Green, microtubule-associated protein 2, neuronal dendritic marker), and anti-glutamate (Red, glutamatergic neuron marker) antibodies. BDNF increased the number of neurites of glutamatergic neuron, but it was inhibited by pretreatment with DEX (DEX, a synthetic glucocorticoid, 10 μM). DEX was applied at DIV1, and BDNF (100 ng/ml) was applied at DIV2 in the presence or absence of DEX. **B.** BDNF-increased synapsin I (presynaptic) and NR2A (postsynaptic) proteins was inhibited by DEX. Expression of TUJ1 (class III ß-tubulin), a neuronal marker, was not changed. **C.** pERK1/2 (phosphorylated ERK1/2) and total ERK1/2 at 6 hours after BDNF application with or without DEX pretreatment. BDNF-stimulated ERK1/2 activation was decreased by DEX.

development may decrease neuronal network activity after neurons mature, which may lead to a decrease in the activity-dependent production and release of BDNF. BDNF mRNA expression, containing exon II and IV of the hippocampus and prefrontal cortex, was decreased in adult rats administered chronic corticosterone (Dwivedi et al., 2006). However, an increase of BDNF expression in chronically separated infants was reported (Nair et al., 2007). Duration and potency of the stress may regulate the expression level of splice variants for BDNF mRNA in each brain region (Nair et al., 2007; Cirulli et al., 2009).

Correlation between BDNF function and steroid-hormonal action

Another steroid hormone, estrogen, is well known for its relation to BDNF function. A number of reports suggest that estrogen increases BDNF expression (reviewed by Sohrabji and Lewis, 2006). Moreover, estrogen and BDNF may share common signaling pathways, including MAPK/ERK1/2, PI3K, and PLCγ pathways, as both factors have a similar impact on neuronal cells, including facilitation of neuronal plasticity and neuroprotection (Scharfman and MacLusky, 2006). Thus, there is a possibility that both BDNF and estrogen enhance these signaling pathways cooperatively. In contrast to estrogen, glucocorticoid may have a negative influence on BDNF function by inhibiting the BDNF/TrkB signaling. Recently, we have found that chronic exposure to DEX suppresses BDNF-induced glutamate release via weakening the activation of PLCγ/Ca^{2+} signaling in cultured cortical neurons (Numakawa et al., 2009). The GR interacts with TrkB, and the TrkB-GR interaction was reduced due to the decline in GR expression following DEX treatment. In our system, BDNF-dependent binding of PLCγ to TrkB was diminished by DEX. SiRNA transfection for a decrease in endogenous GR mimicked the inhibitory action of DEX. Conversely, the DEX-inhibited PLCγ signaling was recovered by GR overexpression. Another group also showed an essential interaction of GR to a receptor for transducing the cytosolic signal in a T-cell receptor on immune T-cells (Lowenberg et al., 2007). Similarly another receptor kinase, FMS-like tyrosine kinase 3, was also found to interact with GR (Asadi et al., 2008). GR was detectable in the lipid raft on the plasma membrane (Jain et al., 2005; Matthews et al., 2008), therefore, GR may take part in the various signal transductions via regulation of activity of membrane receptors.

Interestingly, acute glucocorticoid exposure induces the phosphorylation of TrkB and promotes neuronal survival (Jeanneteau et al., 2008). The phosphorylation of TrkB peaks at 5 hours and gradually decreases after glucocorticoid exposure. Indeed, activation of TrkB, PLCγ, Akt, and ERK has been shown to increase by short-term application of glucocorticoid, reaching the maximum level at 2–4 h after the application in our cortical cultures. However, GR expression level was not altered by such a short glucocorticoid exposure (Numakawa et al., 2009). Jeanneteau et al. (2008) suggests that the activation of TrkB is achieved through regulation of unknown genes, as TrkB was not activated with a mutant GR that lacks the DNA binding domain. Taken together GR may have two 'faces'; the first as a transcription factor and the second as an adaptor molecule of a membrane receptor, such as TrkB. Because long-lasting exposure of glucocorticoid alters the expression level of its receptor, GR, the duration of glucocorticoid exposure may be important when interpreting the interaction between glucocorticoid/GR action and BDNF/TrkB signaling.

Regulation of BDNF expression

BDNF is broadly expressed in the developing and adult mammalian brain, being especially abundant in the hippocampus, cerebral cortex, cerebellum and amygdala. Expression levels of BDNF are dramatically increased during the first few weeks of postnatal development in rodents (Ernfors et al., 1990; Hofer et al., 1990; Conner et al., 1997; Yan et al., 1997). Intracellular BDNF expression is found predominantly in glutamatergic neurons, not in interneurons or astrocytes, under physiological conditions (although its expression can be detected in cultured astrocytes) (Zafra et al., 1992; Yoshimoto et al., 1995; Cellerino et al., 1996; Rocamora et al., 1996; Gorba and Wahle, 1999). The BDNF gene is comprised of at least eight promoters, each of which initiate transcription of alternative 5' exon spliced onto a common 3' exon, encoding the entire open reading frame of the BDNF protein. Additionally, BDNF mRNA has two distinct polyadenylation sites resulting in production of distinct populations of mRNA, with either short or long 3' untranslated regions (3' UTRs). At least 18 distinct transcripts are generated through the complexity of the BDNF gene (Liu et al., 2006; Aid et al., 2007). Possible explanations for the presence of multiple mRNA variants generating the same protein are: different sequences of mRNA show different distributions and/or translational efficacies, and different transcript expressions are regulated by different stimuli. Indeed, dendritic targeting of BDNF mRNA stimulated by neuronal activity has been reported (Tongiorgi et al., 1997; Tongiorgi, 2008). An et al., recently demonstrated that BDNF mRNA with long 3' UTR is selectively transported to dendrites of hippocampal neurons, and BDNF translated from this transcript is required for spine morphology and synaptic plasticity in dendrites (An et al., 2008).

Neuronal activity regulates the transcription of a large set of genes, many of which encode proteins that modify synaptic function and activity-dependent adaptation to neuronal responses that are believed to be critical for the refinement of neuronal connections

during development and for mature brain functioning. As expected, expression of BDNF in neurons is correlated with neuronal activity. BDNF mRNA level is rapidly increased by seizure activity in the hippocampus and the cerebral cortex (Castrén et al., 1989; Ernfors et al., 1991). Down-regulation of BDNF mRNA was observed in the visual cortex of dark-reared rats by blockade of visual input (Castrén et al., 1992). In cultured neurons, depolarization induced by glutamate or high concentrations of potassium increased the levels of BDNF mRNA, while a blockade of neuronal activity with γ-aminobutyric acid (GABA) decreased such levels (Lindholm et al., 1994; Berninger et al., 1995). It has also been shown that BDNF expression is regulated by intracellular Ca^{2+} and CREB, a key transcription factor that mediates stimulus-dependent transcription (Shieh et al., 1998; Tao et al., 1998). Greenberg and colleagues have demonstrated the importance of the activity-dependent component of BDNF gene transcription for inhibitory synapse development. They introduced a subtle mutation into the mouse BDNF gene that blocks the ability of CREB to bind BDNF promoter IV. These mice demonstrate impairments of the sensory experience-dependent induction of BDNF expression and fewer inhibitory synapses in the cortex (Hong et al., 2008). Regulation of BDNF transcription is also putatively involved in the pathophysiology and treatment of psychiatric disorders. Recent studies have shown that methylation in a BDNF promoter region affects the regulation of BDNF expression. DNA methylation regulates mRNA expression and typically occurs on cytosine residues in which the cytosine is followed by a guanine residue (CpGs) in vertebrates. Methyl-CpG–binding protein 2 (MeCP2) is a member of the methyl-CpG–binding protein family that functions as a transcriptional regulator. Mutations in MeCP2 were found in many cases of Rett syndrome, an X-linked disorder characterized by arrested neurological development and subsequent cognitive and motor dysfunction. Originally, MeCP2 was characterized as a transcriptional repressor for many genes including BDNF. Indeed, MeCP2 binds to a BDNF promoter and represses expression of BDNF mRNA in cultured neurons (Chen et al., 2003). However, BDNF was down-regulated both at the mRNA and protein levels in MeCP2-null mice (Chang et al., 2006). The more recent study of Chahrour et al. suggests a potential explanation for the MeCP2 function by demonstrating the interaction between MeCP2 and CREB (Chahrour et al., 2008). Using chromatin immunoprecipitation, they verified the association of MeCP2 with CREB at a few target genes in the hypothalamus. Interestingly, MeCP2 functions as both an activator and a repressor, depending on whether it is associated with CREB or not, respectively. Their finding may settle irreconcilable data on the relationship between MeCP2 and BDNF transcription and provide future therapeutic strategies for Rett syndrome. In addition, social defeat stress resulted in a sustained suppression of BDNF transcription through histone methylation, whereas chronic (but not acute) imipramine treatment was shown to restore BDNF synthesis through histone acetylation (Tsankova et al., 2006). Lithium and valproic acid (VPA), two primary drugs used to treat bipolar mood disorder, selectively increased the levels of exon IV containing BDNF mRNA, as well as the activity of BDNF promoter IV. Lithium and VPA affect the BDNF promoter through inhibition of glycogen synthase kinase-3 (GSK-3) and histone deacetylase (HDAC), respectively (Fukumoto et al., 2001; Yasuda et al., 2009).

Mechanisms of BDNF secretion

BDNF is translated at the rough endoplasmic reticulum (ER) as a precursor protein (proBDNF: 32 kDa) which is subsequently cleaved proteolytically to produce the mature form of BDNF (13 kDa). Synthesized proBDNF is moved to the Golgi apparatus and accumulated in membrane stacks of the trans-Golgi network (TGN). Finally, BDNF-containing vesicles bud off from the TGN and are then transported to the secretion sites. Depending on whether the secretion occurs spontaneously or in response to neuronal activity, neurotrophin secretion is classified into "constitutive" or "regulated" pathways, respectively. BDNF appears to be sorted primarily into the regulated pathway in hippocampal neurons (Goodman et al., 1996; Farhadi et al., 2000; Mowla et al., 2001). In the regulated pathway, transport of BDNF-containing vesicles to either axon terminals or dendrites along microtubules has been reported (Kohara et al., 2001; Kojima et al., 2001; Gartner and Staiger, 2002; Adachi et al., 2005), although the specific sites for BDNF secretion have not been elucidated.

Recent studies have revealed some of the details of BDNF vesicular sorting. The pro-region of BDNF has been implicated as a regulator of BDNF sorting to secretory vesicles. A single nucleotide polymorphism (SNP) was identified at amino acid 66 (Val66Met) in the sequence encoding the pro-region of BDNF. Egan and colleagues reported that the met allele was associated with decreased episodic memory and abnormal hippocampal activation in humans. Interestingly, met-BDNF-GFP in cultured hippocampal neurons failed to localize to secretory granules for transport to synapses. Lower secretion of met-BDNF-GFP compared with met type was due to the impairment of BDNF sorting (Egan et al., 2003). NT-4, which is rarely sorted into secretory vesicles, was redirected to sort into secretory vesicles by fusing to the pro-region of BDNF (Brigadski et al., 2005). However, the pro-region of BDNF alone fused to GFP was not sufficient to induce sorting. This data suggests that the pro-region of BDNF contains important determinants for vesicular targeting, although additional targeting sequences in the mature region of BDNF are also required. Furthermore, binding of BDNF to lipid-

raft-associated sorting receptor carboxypeptidase E (CPE) in the TGN appears to be important for sorting (Lou et al., 2005). CPE knockout mice demonstrate a lack of BDNF secretion through the regulated pathway. Interestingly, sortilin, a trans-membrane protein, has also been implicated in the sorting of proBDNF to secretory granules (Chen et al., 2005). Sortilin is distributed on the membrane of secretory granules and interacts specifically with the pro-region of proBDNF. Interestingly, a truncated form of sortilin was not able to sort proBDNF into the secretory vesicles. The involvement of Ca^{2+}-dependent activator protein for secretion 2 (CAPS2) for BDNF secretion was also reported. CAPS2, a secretory granule-associated protein, is abundant at the parallel fiber terminals of granule cells in the cerebellum. CAPS2 knock-out mice demonstrate autistic-like behavioral phenotypes in addition to deficient release of BDNF and NT-3 (Sadakata et al., 2007). Importantly, the human CAPS2 gene is located on chromosome 7q31.32, within a critical autism susceptibility locus 1.

It is still controversial as to where and how pro-neurotrophins are processed into mature neurotrophins in the CNS. Originally, it had been thought that pro-neurotrophins are prototypically cleaved by furin and pro-protein convertases (PCs) in the TGN or in secretory granules before secretion (Seidah et al., 1996; Matsumoto et al., 2008). However, recent studies have indicated that a considerable amount of BDNF is secreted in the pro-form from neurons. Released proBDNF is subsequently processed to mature BDNF extracellularly by proteases such as plasmin or matrix metalloproteinases (Pang et al., 2004; reviewed by Lu et al., 2005; Yang et al., 2009). A possible link between Huntington's disease (HD) and BDNF functions has also been reported. HD is an autosomal dominant neurodegenerative disease characterized by relatively selective degeneration of striatal neurons which leads to psychiatric, cognitive and motor dysfunction. Polyglutamine expansion (polyQ) in the protein huntingtin (htt) is thought to be the principal mechanism for neuronal toxicity in HD. Interestingly, wild-type htt appears to play two roles: One as a transcription factor to facilitate expression of BDNF (reviewed by Zuccato and Cattaneo, 2007) and the other as a regulator of BDNF-containing vesicle transport. Mutant (PolyQ)-htt perturbs post-Golgi trafficking of BDNF in the regulated secretory pathway, while wild-type htt enhances trafficking (Gauthier et al., 2004; del Toro et al., 2006). Further, axonal transport of BDNF-containing vesicles is disrupted in cultured striatal and hippocampal neurons but not in cortical neurons (Her and Goldstein, 2008). These findings suggest that mutation of htt reduces levels of BDNF in the striatum by inhibiting gene expression and perturbing axonal transport of BDNF-containing vesicles to the striatum. Molecular mechanisms of BDNF secretion have been uncovered by an elegant series of studies by Lessmann and colleagues. The activity-dependent postsynaptic secretion of BDNF critically depends on intracellular Ca^{2+} concentration. The Ca^{2+} increase is through ionotropic glutamate receptors, voltage-gated Ca^{2+} channels (VDCC), and the internal Ca^{2+} stores. Furthermore, activation of CaMKII and PKA contribute to BDNF secretion, although Trk signaling and activation of Na^{+} channels are not required (Hartmann et al., 2001; reviewed by Lessmann et al., 2003; Kolarow et al., 2007). Recent reports suggest that the Golgi apparatus is distributed in dendritic shafts as well as the cell soma, and has even been identified as a local BDNF secretory pathway in dendrites (Horton and Ehlers, 2003; Horton et al., 2005). Taking into consideration the possibility of the local translation of BDNF in dendrites and spines (Zhang and Poo, 2002; Tanaka et al., 2008), further studies need to reveal the complex and dynamic secretory systems of BDNF.

The fate of secreted BDNF

BDNF causes many cellular responses after binding to and activating TrkB. Mainly, two possible mechanisms occur in order to facilitate intracellular signal transduction after TrkB activation (reviewed by Nagappan and Lu, 2005; reviewed by Ibáñez, 2007). The first involves internalization of BDNF-bounded TrkB followed by axonal transport of "signaling endosomes." The second involves lateral movement of TrkB from the extrasynaptic membrane to lipid rafts to create a "signaling platform," as mentioned above in this review. The "signaling endosomes" are involved in BDNF-induced survival and morphological change, which require a de novo transcriptional reaction. Long-range travel of "signaling endosomes" from the distal neurites (where BDNF acts) to the cell body is essential for this type of BDNF-dependent biological action. Endocytosis of and retrograde transport of ligand/Trk complexes induces the activation of downstream signaling, including the MAPK/ERK1/2, PI3K and PLCγ pathways (reviewed by Yano and Chao, 2004; reviewed by Lu, 2003; reviewed by Zweifel et al., 2005; reviewed by Bronfman et al., 2007). Several lines of experiments concerning NGF signaling indicate that the signaling endosomes are generated in a clathrin-dependent manner (Grimes et al., 1996; Howe et al., 2001), or require pincher-mediated endocytosis (Shao et al., 2002; Valdez et al., 2007). Dynein motor complex- and microtubule-dependent transport are also thought to be responsible for the Trk-mediated retrograde signals. A recent study indicates that IC-1B, a neuron-specific isoform of the intermediate chain (IC) of dynein, preferentially binds to and transports the TrkB signaling endosomes, while the ubiquitously expressed IC-2C isoform does not (Ha et al., 2008). Furthermore, a requirement of axonal ERK5 and PI3K activation as the retrograde survival signal has been reported (Kuruvilla et al., 2000; Watson et al., 2001). Surprisingly, in cultured dorsal root ganglion (DRG) neurons, newly synthesized CREB in the axons

stimulated by NGF travels via retrograde transport with appropriate signaling endosomes and is required for NGF-dependent retrograde survival (Cox et al., 2008). Activated CREB was co-localized with pTrkA and pERK5, suggesting the involvement of CREB and ERK5 in the TrkA signaling endosome. This data indicates that these downstream signaling molecules participate in the Trks signaling endosomes (reviewed by Cosker et al., 2008). A p75-dependent signal is also generated through the retrograde-transported endosomes in sensory neurons (Curtis et al., 1995; Deinhardt et al., 2006). Some studies show that p75 is cleaved in a ligand-dependent manner, and the resulting intracellular fragment induces intracellular signaling (Kanning et al., 2003; Kenchappa et al., 2006). As there are a number of interacting factors, p75 may undergo different processing and have different signaling mechanisms depending on the type of stimulus or cell it is involved with. Recent studies have shed light on a new aspect of the fate of secreted BDNF: "recycling." Canossa and colleagues have shown that exogenously applied BDNF was internalized by hippocampal neurons via a TrkB-dependent mechanism and rapidly becomes available for activity-dependent secretion. The recycled BDNF played the same role as newly synthesized BDNF in mediating the late-phase LTP (Santi et al., 2006). Interestingly, newly synthesized BDNF in neurons after theta-burst stimulation (TBS) is secreted in proBDNF form and is then rapidly internalized by astrocytes in cortical slices via the formation of a complex with p75 and subsequent clathrin-dependent endocytosis. The endocytosed proBDNF is resecreted via a SNARE-dependent mechanism in astrocytes (Bergami et al., 2008).

Concluding remarks

BDNF plays a critical role in determining the fate of various CNS neurons. BDNF induces many neuronal responses through activation of TrkB, or other receptors such as p75. In general, BDNF maintains dual roles: Firstly, BDNF promotes positive effects in the brain, including promotion of neuronal survival, maintenance of LTP, etc. via TrkB receptors. Secondly, and conversely, BDNF generates negative effects through its induction of apoptosis, depression of neuronal plasticity, etc. via p75 receptors. Recent high-impact discoveries have been made: Lessmann and colleagues have illustrated the activity-dependent secretion of neurotrophins (Hartmann et al., 2001; Lessmann et al., 2003), while Nagappan et al. have discovered that both proBDNF and mature BDNF are indeed secreted from neurons (Nagappan et al., 2009; Yang et al., 2009). It has also been found that high affinity interactions of pro-neurotrophins with p75 have contributed to apoptosis via p75 activation (Lee et al., 2001). Remarkably, roles of BDNF have been implicated in the pathophysiology of various brain diseases, including Alzheimer's disease, Parkinson's disease, and Huntington's disease (reviewed by Mattson 2008). In addition, it is possible that BDNF is involved in several psychiatric disorders, including schizophrenia, bipolar disorder, and major depression (Knable et al., 2004; Gervasoni et al., 2005; Karege et al., 2005). There are many different ways that BDNF acts - constitutive vs. regulated secretion, dendritic vs. axonal secretion sites, pro- vs. mature forms, autocrine vs. paracrine secretion (Acheson et al., 1995; Marini et al., 1998), and TrkB vs. p75 receptors. Furthermore, there are several signaling pathways stimulated by BDNF, depending on the molecular and cellular context. In the future, precise understanding of intracellular and extracellular actions of BDNF will further elucidate biological systems and, more importantly, will help develop new therapeutic targets for psychiatric and neurodegenerative diseases.

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