Electroconvulsive seizure, but not imipramine, rapidly up-regulates pro-BDNF and t-PA, leading to mature BDNF production, in the rat hippocampus

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Abstract

Electroconvulsive therapy is the most effective treatment for antidepressant-resistant depression, although its mechanism has not been fully elucidated. Previous studies have demonstrated that electroconvulsive seizures (ECS) induce expression of brain-derived neurotrophic factor (BDNF) in the rat hippocampus. However, in contrast with mature BDNF (mBDNF) known to have antidepressant effects, its precursor (pro-BDNF) has harmful effects on neurons. We therefore hypothesized that efficient processing of pro-BDNF is a critical requirement for the antidepressant effects of ECS. We found that single administration of ECS rapidly increased not only hippocampal levels of pro-BDNF but also those of prohormone convertase 1 (PC1) and tissue-plasminogen activator (t-PA), which are proteases involved in intra- and extracellular pro-BDNF processing, respectively. Interestingly, pro-BDNF and t-PA levels were increased in hippocampal synaptosomes after single ECS, suggesting their transport to secretory sites. In rats receiving 10-d repeated ECS, accumulation of pro-BDNF and a resultant increase in mBDNF levels were observed. While t-PA levels increased and accumulated following repeated ECS, PC1 levels did not, suggesting that intracellular processing capacity is limited. Finally, chronic administration of imipramine significantly increased mBDNF levels, but not pro-BDNF and protease levels, indicating that the therapeutic mechanism of imipramine differs from that of ECS. Taken together, these results suggest that, while intra- and extracellular proteases are involved in pro-BDNF processing in single ECS, t-PA plays a dominant role following repeated ECS. Such efficient pro-BDNF processing as well as strong induction of BDNF expression may contribute to the antidepressant effects of ECS.

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Introduction

Epidemiological studies indicate that the current lifetime prevalence of major depressive disorder (MDD) exceeds 10% (Kessler et al. 1994, 2003) and that at least 10% of patients with MDD fail to have a favourable response to antidepressant drugs (Keller & Boland, 1998; Keller et al. 1992). Due to the high prevalence of MDD, it is clinically important to acquire a more thorough understanding of the pathophysiology of this condition. According to the usual treatment algorithm for MDD, electroconvulsive therapy (ECT) is recommended for drug-resistant depressed patients (UK ECT Review Group, 2003). Although numerous studies have examined the therapeutic actions of antidepressant drugs and ECT, the underlying mechanisms have not been fully uncovered.

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, plays an important role...
in neuronal survival and plasticity (Bibel & Barde, 2000) and has been postulated to be involved in the mechanism of action of antidepressants (Duman, 2002; Nestler et al. 2002). Chronic stress decreases the expression of BDNF in the rat hippocampus (Smith et al. 1995). A reduction of BDNF expression in the dentate gyrus of the rat hippocampus induces depression-like behaviour (Taliaz et al. 2010) and attenuates the action of antidepressants (Adachi et al. 2008). BDNF administration produces antidepressant-like effects in animal models of depression (Siuciak et al. 1997). Interestingly, chronic administration of antidepressant drugs and repeated electroconvulsive seizure (ECS) facilitate the transcription and translation of BDNF in the rat hippocampus (Altar et al. 2003; Nibuya et al. 1995). ECS is reported to improve a depressive-like behaviour in a rat model (Li et al. 2006). A recent study suggests the involvement of the dorsal, but not the ventral, hippocampus in the ECS-induced BDNF increase (Gersner et al. 2010). Consistent with these findings in animal models, BDNF levels are decreased in post-mortem hippocampus of depressed patients and administration of antidepressant drugs leads to restoration of BDNF levels (Chen et al. 2001). Taken together, these findings suggest that BDNF up-regulation in the hippocampus in response to antidepressant drugs and ECS may be closely involved in the improvement of various symptoms in patients with MDD (Bocchio-Chiavetto et al. 2006).

BDNF protein is biosynthesized as a precursor (pro-BDNF) that is post-translationally processed to mature BDNF (mBDNF). mBDNF is then secreted in an activity-dependent manner to exert antidepressant effects through the TrkB receptor. In brain neurons, the processing of pro-BDNF occurs rapidly in the intracellular compartment (Matsumoto et al. 2008; Mowla et al. 2001), where prohormone convertase 1 (PC1) and furin are thought to be involved (Lessmann et al. 2003). Importantly, the expression of BDNF and PC1 in the hippocampus is increased upon neuronal activity stimulated by pilocarpine, a chemical that induces seizures (Marcinkiewicz et al. 1997). However, recent studies demonstrate that pro-BDNF induces neuronal apoptosis through the p75 neurotrophin receptor (p75NTR; Lu et al. 2005). It is currently hypothesized that pro-BDNF/p75NTR signalling is involved in the pathophysiology of depression (Martinowich et al. 2007). Therefore, efficient processing of pro-BDNF would seem to be critical for the antidepressant actions of BDNF, although this hypothesis remains to be proven.

Considering the negative impact of pro-BDNF on neurons and the antidepressant effects of ECS, signalling via mBDNF, not pro-BDNF, would be activated upon ECS. However, since most studies measuring BDNF levels have used an enzyme-linked immunoassay (ELISA), which cannot distinguish between pro-BDNF and mBDNF, the form of BDNF that is increased upon ECS remains unknown. Since ECS leads to a rapid and large increase in hippocampal BDNF mRNA levels (increased by ~10-fold; Nibuya et al. 1995), massive amounts of pro-BDNF may be synthesized, leading to exhaustion of the capacity of intracellular pro-BDNF processing. If this is the case, additional extracellular pro-BDNF processing machinery may be needed in order for the antidepressant effects of ECS to be fully exerted. Of the known extracellular proteases, tissue-plasminogen activator (t-PA), a serine protease converting plasminogen to plasmin (the active protease), is of particular interest. Although the t-PA/plasmin system is known to play a key role in the fibrinolysis of blood clots, t-PA is also expressed in brain neurons and regulates synaptic plasticity (Qian et al. 1993). Interestingly, this system was recently suggested to be involved in extracellular pro-BDNF processing in the hippocampus (Pang et al. 2004). However, the antidepressant actions of t-PA remain largely unknown.

Since a majority of depressed patients resistant to antidepressant drugs show a favourable response to ECT, the therapeutic action of ECT may be different from that of antidepressant drugs. It is therefore important to elucidate the mechanistic differences underlying these respective antidepressant effects, which may facilitate the development of new therapeutic strategies. While many antidepressant drugs, including imipramine, increase BDNF expression (Duman & Monteggia, 2006), no study has yet examined whether pro-BDNF processing activity is differentially affected by ECS and antidepressant drugs.

In this study, we (1) examined which forms of BDNF (pro-BDNF vs. mBDNF) are increased following ECS administration, (2) determined whether ECS affects the expression of intra- and extracellular protease and (3) compared these effects of ECS with those of the tricyclic antidepressant imipramine.

Materials and method

Animals

Male Sprague–Dawley rats (250–300 g; Charles River, Japan) were group housed (three per cage) in a temperature-controlled environment under a 12-h light/dark cycle (lights on 08:00 hours) with free access to food and water and were given a
1-wk acclimatization period prior to experimental manipulations (shown below). All animal procedures were approved by Hiroshima University Animal Care and Use Committee.

**Electroconvulsive seizure**

After acclimatization, the rats received ECS treatment as described previously but with minor modifications (Nibuya et al. 1995). Briefly, bilateral ECS was administered via spring-loaded ear clip electrodes using a pulse generator (ECT Unit 7801; Ugo Basile, Italy; frequency = 100 pulses/s; pulse width = 0.5 ms; shock duration = 0.5 s; current = 55 mA). This procedure consistently induced a generalized grand mal seizure with characteristic clonic and tonic convulsions. Animals received either a single shock or 10 shocks (once daily) between 09:00 and 10:00 hours. In sham rats, no shock was delivered via the ear clip electrode. In single ECS experiments, the rats were killed by decapitation at 1, 2, 4, 8, 16 and 24 h after ECS treatment to examine temporal changes in mRNA and protein levels of BDNF and proteases. The hippocampi were then collected, frozen immediately in liquid nitrogen and stored at −80 °C until use. In repeated ECS experiments, the hippocampi were collected at 1, 2 and 24 h after the last ECS to examine either the acute effects (1 and 2 h) observed with single ECS administration or the cumulative effects (24 h) of repeated ECS on the expression of BDNF and protease.

**Imipramine treatment**

After acclimatization, the rats received imipramine treatment as described previously (Airan et al. 2007). Animals received daily i.p. injections of imipramine hydrochloride (Sigma-Aldrich, USA; 20 mg/kg.d) or vehicle 0.9% saline (2 ml/kg.d) between 09:00 and 10:00 hours for 14 d. At 2 h after the last injection, the hippocampi were collected.

**Measurement of mRNA levels by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)**

Total RNA from frozen hippocampal tissues was extracted using an Ambion RNAqueous kit (Applied Biosystems, USA) according to the manufacturer’s instructions and single-stranded cDNA was synthesized using the QuantiTect Reverse Transcription kit (Qiagen, Germany), followed by qRT-PCR using ABI PRISM 7900HT sequence detection system (Applied Biosystems). The primers and TaqMan hybridization probes for BDNF were designed using Primer Express software (Applied Biosystems): 5’-CCATAAGGACGGGGAGCTTGTG-3’ (forward), 5’-GAGGCTCAAAGGACCTTGA-3’ (reverse), TaqMan probe, 5’-CAGTCCGTGGGTGATGCTTGC-3’. To determine t-PA, PC1 and furin mRNA expression, TaqMan Gene Expression Assays (Applied Biosystems) were used (Assay ID; t-PA: Rn00565767_m1, PC1: Rn00567266_m1, furin: Rn00570970_m1). Thermal cycling was initiated with an initial denaturation at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of PCR. Each cycle consisted of 15 s melting at 95 °C and 1 min annealing/extension at 60 °C. The levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were determined for normalization using the TaqMan Rodent GAPDH Control Reagents kit (Applied Biosystems). The PCR assay for unknown samples was performed simultaneously with standard mRNA samples prepared from an adult rat hippocampus to construct a standard curve. The relative concentrations of GAPDH and genes in unknown samples were calculated from this standard curve. All standards and samples were assayed in triplicate. The ratio of the concentration of the target gene to that of GAPDH (target gene:GAPDH) in unknown samples was calculated.

**Detection of pro-BDNF and mBDNF by immunoprecipitation/Western blotting**

Immunoprecipitation and Western blotting were performed to detect pro-BDNF and mBDNF as described previously (Matsumoto et al. 2008). Briefly, lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.2% Na deoxycholate) including 1x complete protease inhibitors (Roche Diagnostics, Germany) was added to frozen hippocampal tissues (10:1 w/v), followed by ice-cold sonication. Samples were then centrifuged (30 min, 4 °C, 17 360 g) and the supernatants were collected. Protein concentration was determined by BCA protein assay (Thermo Scientific, USA). Immunoprecipitation of lysate proteins (100 μg) was performed with mouse monoclonal anti-BDNF antibodies, mAb9 (0.5 μg/sample; a gift from Dr Y.-A. Barde, University of Basel, Switzerland; Kolbeck et al. 1999) in the presence of 10 μl Dynabeads Protein G (Invitrogen, Norway) for 2 d at 4 °C. Immunoprecipitates were separated by electrophoresis using NuPAGE 4–12% gradient SDS-polyacrylamide gels (Invitrogen, USA) and proteins were transferred onto a nitrocellulose membrane (GenScript, USA) using transfer buffer solution (39 mM glycine, 48 mM Tris-base, 0.037% SDS, 20% methanol). The proteins...
were probed with rabbit polyclonal anti-BDNF antibodies N20 (sc-546; Santa Cruz Biotechnology, USA) using an IPWestern Kit (GenScript), according to the manufacturer’s instructions. Immunoreactive bands were detected using a chemiluminescence system (Amersham ECL-plus; GE Healthcare, UK). Signal intensities were measured using ImageJ software (NIH, USA). To determine the tissue concentration of pro-BDNF and mBDNF, known amounts of recombinant purified mBDNF (a gift from Dr Y.-A. Barde) were used as a reference.

**Measurement of t-PA activities by zymography**

Gel zymography was performed as previously reported (Nagai et al. 2004). Hippocampal tissue lysates (40 µg) or synaptosome lysates (see below, 100 µg) were subjected to gel electrophoresis using 10% polyacrylamide SDS gels containing casein (1 mg/ml; Sigma-Aldrich, USA) and plasminogen (15 µg/ml; Chromogenix, Sweden). After electrophoresis, SDS was extracted from the gel with 2.5% Triton X-100 for 30 min twice and the gel was incubated was extracted from the gel with 2.5% Triton X-100 for 30 min twice and the gel was incubated for 5 h (hippocampal tissue lysates) or 20 h (synaptosome lysates) with 0.1M Tris-HCl (pH 8.0) at 37°C, followed by Simply Blue staining (Invitrogen). The intensity of the band at around 65 kDa, where recombinant t-PA was detected, was measured using ImageJ software.

**Rat hippocampal synaptosome preparation**

Rat hippocampal synaptosomes were prepared according to a well-established method using Percoll (GE Healthcare, Sweden; Dunkley et al. 2008). In this method, not only synaptic vesicles but also large dense-core vesicles (into which BDNF is packed) are collected in synaptosome fractions (Dunkley et al. 1988). Briefly, a discontinuous Percoll gradient, comprising layers of 3, 10 and 23% (v/v) Percoll, was prepared. Homogenizing buffer (1 ml; 0.32M sucrose, 1 mM EDTA, 5 mM Tris, pH 7.4) including EDTA-free 1x complete protease inhibitors (Roche Diagnostics, Germany) was added to ~100 mg hippocampal tissue in a loose-homogenizing glass tube, followed by homogenization with 10 strokes by hand. The homogenates were centrifuged at 1000 g for 10 min at 4°C, using an Allegra 64R Centrifuge (Beckman Coulter, USA). The supernatant was collected (S1) and 1.5 ml homogenizing buffer was added to the pellet (P1). After homogenization/centrifugation, the supernatant (S2) was collected. The pooled supernatants (S1 + S2) were diluted with homogenizing buffer to prepare about 4–5 mg/ml protein solution. The diluted solutions (2 ml) was poured over the top of the 3% Percoll layer, followed by centrifugation at 31000 g for 5 min at 4°C. The synaptosomal fraction between the 10 and 23% Percoll layers was collected and washed with sucrose/EDTA buffer (0.32 m sucrose, 1 mM EDTA, 5 mM Tris, pH 7.4). After centrifugation at 2000 g for 30 min at 4°C, the pellets were lysed with the protein lysis buffer indicated above. Because of a low protein yield, synaptosome lysates were pooled together from two rats. In total, four lysates were prepared from eight sham-treated rats and six lysates were prepared from 12 rats that received single ECS. The lysates were analysed by immunoprecipitation/Western blotting or zymography.

**Statistical analyses**

Values are expressed as mean ± S.E.M. Statistical significance was analysed by either Student’s t test or one-way analysis of variance followed by Tukey–Kramer’s post-hoc test. Two-tailed p values of < 0.05 were considered statistically significant.

**Results**

**Levels of BDNF mRNA in response to ECS in the rat hippocampus**

A rapid increase in hippocampal levels of BDNF mRNA after ECS administration was previously reported in a study using in situ hybridization (Nibuya et al. 1995). To confirm this finding and further examine temporal changes in BDNF mRNA levels after ECS administration, we collected hippocampal tissues at 1, 2, 4, 8 and 24 h after single ECS administration and performed qRT-PCR. A single administration of ECS significantly (F<sub>5,58</sub>= 262.35, p < 0.001) increased the levels of BDNF mRNA within 1 h (p < 0.001; Tukey–Kramer’s post-hoc test) and the maximum increase was found at 2 h [p < 0.001; Tukey–Kramer’s post-hoc test (6.5-fold that of the sham group)] after ECS. Thereafter, the levels returned to basal levels within 24 h (Fig. 1a). Repeated administration of ECS also significantly (F<sub>5,57</sub>= 121.31, p < 0.001) increased the levels of BDNF mRNA 1 and 2 h (p < 0.001, p < 0.001, respectively; Tukey–Kramer’s post-hoc test) after the last ECS (Fig. 1b). A similar pattern of changes in the levels of BDNF mRNA was observed at 1 and 2 h after the last ECS in rats receiving ECS repeatedly. The levels returned to basal levels 24 h after the last ECS administration (Fig. 1b), suggesting little accumulative effects of repeated ECS administration.
To examine whether ECS changes the levels of pro-BDNF and mBDNF, immunoprecipitation/Western blotting was performed. A single administration of ECS significantly increased the levels of pro-BDNF ($F_{6,55} = 55.87$, $p < 0.001$) within 2 h ($p < 0.001$; Tukey–Kramer’s post-hoc test) after the treatment and the levels were sustained for at least 6 h, followed by a

**Levels of pro-BDNF and mBDNF protein in response to ECS**

The effect of ECS on brain-derived neurotrophic factor (BDNF) expression in the rat hippocampus is depicted in Fig. 1. (a) Single administration of ECS rapidly increased BDNF mRNA as compared to sham rats, followed by a gradual return to basal levels. (b) In 10-d repeated ECS, BDNF mRNA was rapidly increased after the last ECS administration. Values are expressed as mean ± S.E.M. ($n = 9–10$). (c, d) The effect of ECS on hippocampal levels of pro-BDNF and mature BDNF (mBDNF) was determined by immunoprecipitation/Western blotting. Bands indicated by an arrow were derived from the light chain of antibodies used in immunoprecipitation. Quantification of these results is shown in (e–h). (e) Single ECS increased pro-BDNF levels rapidly. (f) A similar increase in pro-BDNF levels was observed for repeated ECS. (g) Significant changes in mBDNF levels were not detected for single ECS. (h) mBDNF was clearly increased by repeated ECS. Values are expressed as mean ± S.E.M. ($n = 8$). * $p < 0.01$, ** $p < 0.05$ compared to sham-treated controls (Tukey–Kramer’s post-hoc test).
Levels of intra- and extracellular protease mRNA in response to ECS

We next examined whether ECS affected the expression of intra- (furin and PC1) and extracellular proteases (t-PA) known to be involved in pro-BDNF processing in the hippocampus (Lessmann et al. 2003; Pang et al. 2004). Furin mRNA was very weakly but significantly increased at 2 h (p < 0.001, p = 0.01, respectively; Tukey-Kramer’s post-hoc test) after single ECS (F(4, 45) = 24.39, p < 0.001; 1.4-fold vs. sham) or repeated ECS (F(4, 39) = 5.68, p = 0.003 (1.2-fold vs. sham); Fig. 2a, b). By contrast, PC1 mRNA was more clearly increased within 1 h (p < 0.001; Tukey-Kramer’s post-hoc test) after single ECS [F(4, 45) = 63.37, p < 0.001; (1.8-fold vs. sham); Fig. 2c]. However, PC1 mRNA did not increase after repeated ECS (F(4, 39) = 9.37, p < 0.001; Fig. 2d). In line with these mRNA results, PC1 protein levels were increased by single ECS, but not after repeated ECS administration (see Supplementary Fig. S2c). ECS did not change the levels of furin protein (see Supplementary Fig. S2a, b).

Next, we focused on t-PA. A similar rapid increase in the levels of t-PA mRNA was observed at 1 h (p < 0.001; Tukey-Kramer’s post-hoc test) after single ECS [F(4, 45) = 62.63, p < 0.001; (2.2-fold vs. sham); Fig. 2e]. Unlike with PC1 (Fig. 2d), an acute increase in t-PA levels was observed in rats receiving repeated ECS (F(4, 39) = 26.26, p < 0.001; Fig. 2f). The increased levels of t-PA mRNA were sustained at 2 h (p < 0.001 for both; Tukey-Kramer’s post-hoc test) after single and repeated ECS, followed by a decline to basal levels within 24 h (Fig. 2e, f). The peak increase of t-PA mRNA was found earlier than that of BDNF mRNA in response to single as well as repeated ECS. These results suggest that not only intracellular protease PC1 but also extracellular t-PA may be involved in efficient processing of pro-BDNF produced by ECS.

Levels of pro-BDNF and mBDNF in rat hippocampal synaptosomes in response to single ECS

Our results showing the storage of increased levels of pro-BDNF (Fig. 1c–f) prompted us to examine whether pro-BDNF might be transported to synaptic terminals in response to ECS. Therefore, we prepared hippocampal synaptosomal fractions from rats with or without exposure to ECS and examined the levels of pro-BDNF and mBDNF. As shown in Fig. 3, pro-BDNF was clearly detected in lysed synaptosomes 4 h after ECS, while there was almost no detectable pro-BDNF in those from sham rats. On the other hand, mBDNF levels did not change markedly (Fig. 3). This result suggests that pro-BDNF rapidly moves to synaptic terminals by surviving intracellular processing to a certain extent.

Activity of t-PA in the rat hippocampus and synaptosomes in response to ECS

Since pro-BDNF is rapidly transported to synaptic terminals after ECS (Fig. 3), we examined whether t-PA proteins might be increased and then transported to synaptic terminals by ECS. We performed zymography to measure t-PA activities in hippocampal tissue and synaptosome lysates. Single administration of ECS (F(4, 39) = 15.04, p < 0.001) significantly increased the activities of t-PA in hippocampal tissue lysates within 1 h (Fig. 4a), which was earlier than the ECS-induced increase in pro-BDNF levels (Fig. 1c, e). Peak t-PA activity occurred at 1 h (p < 0.001) after ECS (1.6-fold vs. sham) and the increased activities were found even 24 h (p < 0.001) post-ECS (Fig. 4a). The changes in t-PA activity with repeated ECS (F(4, 39) = 10.16, p < 0.001) showed the same tendency (Fig. 4b). These results suggest that t-PA up-regulation precedes ECS-induced production of pro-BDNF. Importantly, in hippocampal synaptosomal lysates, the activity of t-PA was significantly increased 4 h (t = 5.48, d.f. = 6, p = 0.002) after single ECS (1.6-fold) compared to sham rats (Fig. 4c). These results indicate that, like pro-BDNF, t-PA undergoes similar rapid transportation to synaptic terminals as a consequence of ECS.
Effects of chronic imipramine administration on BDNF and t-PA in rat hippocampus

To examine whether the effects of ECS described above might differ from those of antidepressant drugs, we measured the levels of mBDNF and pro-BDNF in response to a single and a 14-d administration of a classical tricyclic antidepressant, imipramine. As a result, single imipramine administration did not increase the levels of both pro-BDNF and mBDNF (see Supplementary Fig. S3a–c). On the other hand, the level of mBDNF in the hippocampus increased slightly compared to the saline-treated control group ($t = 3.10$, d.f. = 14, $p = 0.007$; Fig. 5b, c), while pro-BDNF levels did not change ($t = 0.37$, d.f. = 14, $p = 0.72$; Fig. 5a, c). Unlike ECS, administration of imipramine did not change the levels of furin ($t = 0.64$, d.f. = 16, $p = 0.53$), PC1 ($t = 0.68$, d.f. = 16, $p = 0.51$) or t-PA ($t = 1.30$, d.f. = 16, $p = 0.21$) mRNA 2 h after the last administration of imipramine (Fig. 5d–f). Moreover, accumulation of t-PA activity ($t = 1.54$, d.f. = 14, $p = 0.14$) was not observed (Fig. 5g).
Discussion

The principal findings of this study are the following: (1) both single- and repeated ECS administration rapidly increased the levels of pro-BDNF; (2) the expression of intracellular protease PC1 was correspondingly induced by single ECS, but not by repeated ECS; (3) the activity of t-PA was clearly increased in response to both single- and repeated ECS; (4) pro-BDNF and t-PA were similarly increased in the synaptosomal fraction to some degree upon ECS; (5) chronic treatment with imipramine slightly increased the levels of mBDNF but without producing any changes in the levels of pro-BDNF or the various proteases examined.

A growing body of evidence suggests that BDNF plays a key role in the therapeutic action of antidepressant drugs and ECT. The diverse aspects of BDNF function, such as the enhancement of neurogenesis, neuronal differentiation and synaptogenesis, may be closely involved in these antidepressant effects (Chen et al. 2009; Li et al. 2007; Madsen et al. 2000; Suzuki & Masuda, 1999). In the rat hippocampus, antidepressant drugs and ECS both increased the levels of BDNF mRNA (Nibuya et al. 1995). As expected, BDNF protein levels were increased in the hippocampus upon treatment with antidepressant drugs or ECS (Duman & Monteggia, 2006). However, it was unclear which forms of BDNF were increased. This issue is critical, since pro-BDNF induces neuronal apoptosis through the activation of p75NTR (Lu et al. 2005; Martinowich et al. 2007; Teng et al. 2005). Thus, dysfunction of the pro-BDNF processing machinery...
may have a negative impact on neurons. Nevertheless, the role of pro-BDNF processing enzymes in antidepressant effects had received scant attention. In the present study, we demonstrated that both intracellular and extracellular proteases may be involved in the antidepressant effect of ECS. Because neurotrophins play a pivotal role in the development of the nervous system (Bibel & Barde, 2000), understanding the biosynthesis and processing of neurotrophins is critical for elucidating their involvement in physiological and pathophysiological processes. Because precursor neurotrophins have negative effects on neurons, it is also important to clarify the mechanisms by which the precursors are converted to mature protein. It is well accepted that members of the furin family contribute to the intracellular processing of pro-neurotrophin (Lessmann et al., 2003). Recently, it has been demonstrated that newly synthesized pro-BDNF is rapidly converted to mBDNF in the intracellular compartment of intact brain neurons (Matsumoto et al., 2008). In line with this finding, we observed that single ECS induced the expression of PC1 and furin (Fig. 2a, c). PC1 may predominantly contribute to intracellular processing of pro-BDNF, since the induction of PC1 mRNA expression was stronger and more rapid than the induction of furin. Importantly, single ECS led to an increase in the levels of PC1 protein only, while furin protein levels were unaffected (see Supplementary Fig. S2). This view is supported by previous observations that pilocarpine-induced neuronal activity increased the expression of BDNF and PC1, but not of furin, in the hippocampus (Marcinkiewicz et al., 1997). However, repeated ECS did not induce PC1 at either the mRNA or protein levels (Fig. 2; see also Supplementary Fig. S2), suggesting that the involvement of intracellular proteases may be more evident during the early phase of repeated ECS than during the late phase.

One of the most striking observations in the present study was that single ECS led to an acute (within 2 h) and marked (≥4-fold) increase in pro-BDNF levels (Fig. 1e). The strong increase in pro-BDNF levels might result from stimulation of de novo protein synthesis, since BDNF mRNA was more rapidly increased upon ECS within 1 h (Fig. 1a). However, after the peak increase at 2 h, the mRNA levels decreased rapidly (Fig. 1c), suggesting that the rate of pro-BDNF production was decreased as well. Therefore, if the capacity of intracellular processing of pro-BDNF is high enough, it might be expected that pro-BDNF levels would decrease rapidly, as reported previously (Matsumoto et al., 2008). However, to our surprise, the increased levels of pro-BDNF upon ECS were maintained (but not further increased) for more than 6 h after the initial increase (Fig. 1e), suggesting that the capacity of intracellular processing may be limited and

![Fig. 5. Effect of chronic imipramine (Imi) administration in the rat hippocampus. (a–c) 14-d administration of Imi increased the hippocampal levels of mature brain-derived neurotrophic factor (mBDNF), but not pro-BDNF, compared to saline-treated controls (Sal). Values represent mean ± s.e.m. (n = 8). Bands indicated by an arrow were derived from the light chain of antibodies used in immunoprecipitation (d–f) 2 h after the last administration of Imi, the levels of furin (d) prohormone convertase 1 (PC1) (c) and tissue-plasminogen activator (t-PA) mRNA (f) did not change. (g) No accumulation of t-PA activity was observed. Values represent mean ± s.e.m. (n = 9–10). *p = 0.007 compared to Sal (Student’s t test).](http://ijnp.oxfordjournals.org/)

ECS increases hippocampal BDNF and t-PA
becomes exhausted following ECS. In support of this idea, pro-BDNF was rapidly transported to synaptic terminals (Fig. 3), implying the possibility of pro-BDNF secretion. While pro-BDNF secretion has previously been suggested (Nagappan et al. 2009), there is no clear evidence supporting pro-BDNF secretion in vivo. Our results, obtained using synaptosomes from rats receiving ECS, may uncover the mechanism of pro-BDNF secretion. Although technically very difficult, direct detection of secreted pro-BDNF as well as mBDNF in the brain would further promote our understanding of their secretion.

Considering the negative impact of pro-BDNF on neurons (Lu et al. 2005), if substantial amounts of pro-BDNF are secreted and unprocessed extracellularly, the therapeutic actions of ECS may never be fully achieved. However, we found that the expression of t-PA was clearly induced in response to both single and repeated ECS (Figs 2e,f, 4a,b). In particular, the activity of t-PA in synaptosomes was increased rapidly following single ECS vs. sham treatment (Fig. 4c). These results suggest that unprocessed pro-BDNF and t-PA may be secreted together as a result of ECS, leading to extracellular production of mBDNF, a view supported by previous studies (Pang et al. 2004). Unlike intracellular processing enzymes, t-PA was increased during the entire phase of repeated ECS (Fig. 4a,b). Considering that repeated administration of ECT is necessary for its antidepressant effects, t-PA may play a crucial role. While t-PA is known to be involved in various physiological functions in the brain, including synaptic plasticity in the hippocampus (Qian et al. 1993), little was known concerning its involvement in antidepressant effects. Our findings may shed new light on the role of t-PA in the antidepressant actions of ECS, although further studies examining the precise mechanisms are necessary.

Finally, our findings showing differences in the influence of imipramine and ECS on the biosynthesis of BDNF could be clinically important. Unlike repeated ECS, chronic treatment with imipramine did not change the levels of t-PA (Fig. 5f,g), suggesting little contribution of extracellular protease activity. In line with this finding, imipramine only slightly (although significantly) increased mBDNF, but not pro-BDNF (Fig. 5a–c). Since intracellular proteases did not change (Fig. 5d,e), the basal activity of intracellular proteases may be sufficient to slightly increase mBDNF levels. Further studies examining whether different types of antidepressant drugs affect the extracellular processing of pro-BDNF through t-PA would be informative in this regard.

According to expert consensus guidelines, the therapeutic algorithm of major depression recommends the administration of ECT for severely depressed patients who do not show favourable responses to antidepressants and mood stabilizers, suggesting that the antidepressant action of ECT may be superior to that of drugs prescribed for the treatment of major depression. These findings, together with our results, suggest that both stronger induction of BDNF expression and facilitation of pro-BDNF processing are important for maximizing the efficacy of treatment for depression. Applying this mechanism may facilitate the development of new more effective therapeutic strategies.

In summary, ECS, but not imipramine, affects the expression of pro-BDNF processing enzymes. Due to the limited capacity of intracellular processing activity, extracellular processing via t-PA may be involved to a certain extent. Furthermore, strong and rapid up-regulation of mBDNF, which may be supported by increased t-PA activity, is presumed to be important for the therapeutic action of ECS. On the other hand, a gradual slight increase in mBDNF levels occurs with long-term imipramine treatment and may contribute to the drug’s antidepressant effects. Further studies examining the involvement of t-PA using an animal model of depression are required to fully elucidate the therapeutic action of ECS.

Note
Supplementary material accompanies this paper on the Journal’s website (http://journals.cambridge.org/pnp).

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Statement of Interest
None.
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