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Brain-derived neurotrophic factor can act as a pronecrotic factor through transcriptional and translational activation of NADPH oxidase

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Several lines of evidence suggest that neurotrophins (NTs) potentiate or cause neuronal injury under various pathological conditions. Since NTs enhance survival and differentiation of cultured neurons in serum or defined media containing antioxidants, we set out experiments to delineate the patterns and underlying mechanisms of brain-derived neurotrophic factor (BDNF)–induced neuronal injury in mixed cortical cell cultures containing glia and neurons in serum-free media without antioxidants, where the three major routes of neuronal cell death, oxidative stress, excitotoxicity, and apoptosis, have been extensively studied. Rat cortical cell cultures, after prolonged exposure to NTs, underwent widespread neuronal necrosis. BDNF-induced

neuronal necrosis was accompanied by reactive oxygen species (ROS) production and was dependent on the macromolecular synthesis. cDNA microarray analysis revealed that BDNF increased the expression of cytochrome b_{558}, the plasma membrane-spanning subunit of NADPH oxidase. The expression and activation of NADPH oxidase were increased after exposure to BDNF. The selective inhibitors of NADPH oxidase prevented BDNF-induced ROS production and neuronal death without blocking antiapoptosis action of BDNF. The present study suggests that BDNF-induced expression and activation of NADPH oxidase cause oxidative neuronal necrosis and that the neurotrophic effects of NTs can be maximized under blockade of the pronecrotic action.

Introduction

Survival of central and peripheral neurons largely depends on contact with neurotrophins (NTs)* that are released from their target cells (Levi-Montalcini, 1987; Barde, 1994). The neurotrophic effect of NTs is initiated through binding to TrkA, TrkB, or TrkC, the high affinity NT receptors with tyrosine kinase activity (Kaplan and Miller, 2000; Patapoutian and Reichardt, 2001). The Trk tyrosine kinases activate the small GTP-binding protein Ras, PI-3K, and PLCγ, which play

an important role in survival of a variety of neurons, including cerebellar granule, cortical, hippocampal, sympathetic, and sensory neurons (Borasio et al., 1993; Stephens et al., 1994; Yao and Cooper, 1995; Nobes et al., 1996; Nonomura et al., 1996; Alcantara et al., 1997; Hetman et al., 1999; Atwal et al., 2000). NTs enhance neuronal survival by interfering with programmed cell death or apoptosis in the process of normal development (Barde 1994; Deshmukh and Johnson, 1997). The neuroprotective effects of NTs have been observed in the central neurons subjected to pathological insults. For example, NTs ameliorate degeneration of basal forebrain cholinergic neurons, retinal ganglion neurons, and spinal sensory and motor neurons after axotomy in vivo (Hefti, 1986; Mey and Thanos, 1993; Morse et al., 1993; Yan et al., 1993; Cohen et al., 1994; Friedman et al., 1995). NGF, brain-derived neurotrophic factor (BDNF), and NTs-4/5 can reduce neuronal death after hypoxic-ischemic injury (Shigeno et al., 1991; Beck et al., 1994; Chan et al., 1996). BDNF protects dopaminergic neurons from 1-methyl-

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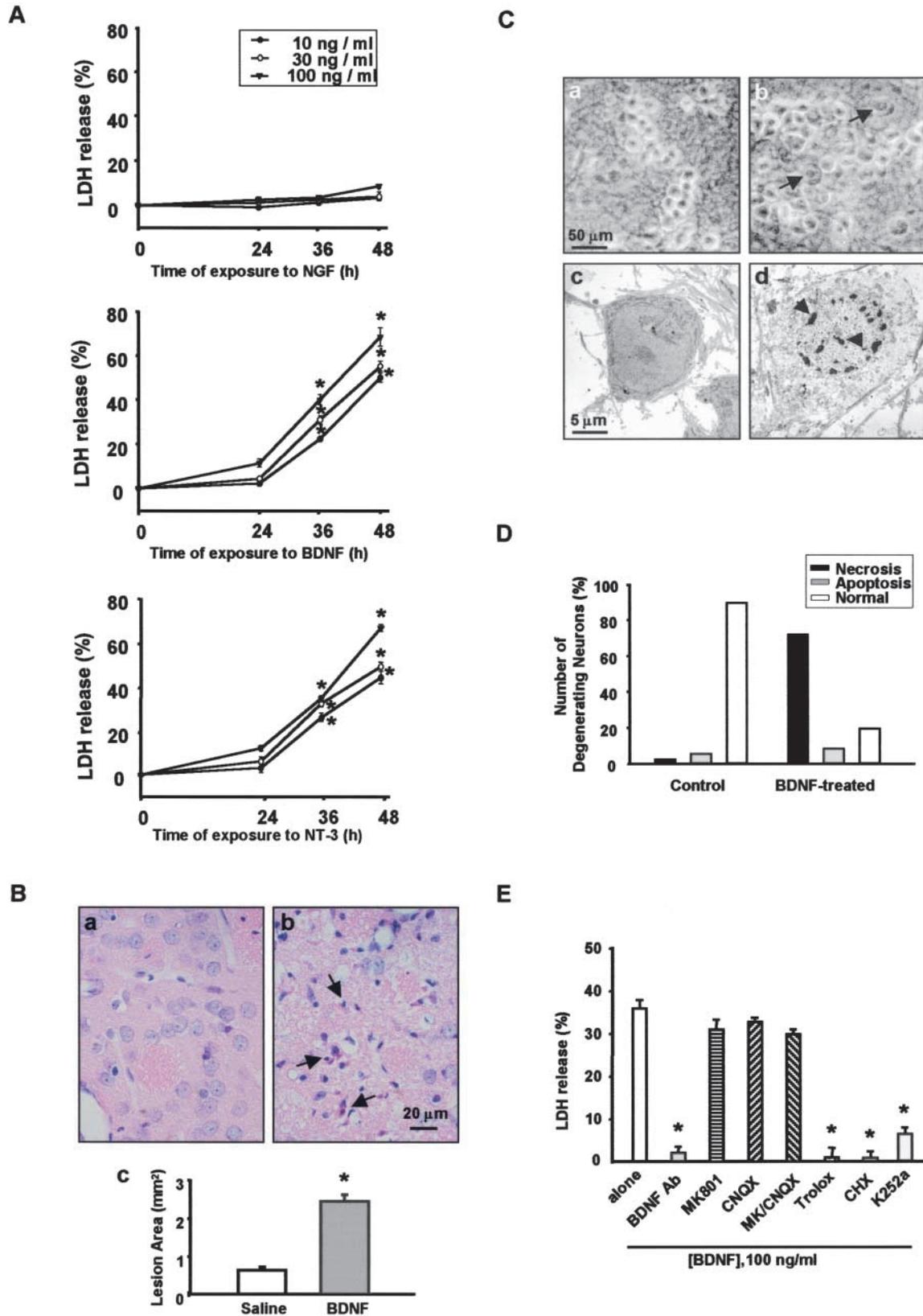
Figure 1.   **Prolonged exposure to NTs produces neuronal cell necrosis in cortical cell cultures.** (A) Mixed cortical cell cultures (DIV 12–15) were continuously exposed to 10, 30, and 100 ng/ml of NGF, BDNF, or NT-3 for the indicated points of time. Neuronal cell death was assessed by measurement of LDH efflux to the bathing medium, mean ± SEM (n = 16 culture wells per condition). *Significant difference from the relevant control (sham washed control) at P < 0.05 using analysis of variance and Student-Neuman-Keuls test. (B) Brain sections were stained with hematoxylin-eosin at 2 d after intrastriatal injections of 5 μl of saline or 5 μg BDNF. Bright field photomicrogrphs showing a representative striatal area 1 mm lateral to the injection site of saline (a) or BDNF (b). Note the pyknotic neurons (arrows) in BDNF-treated

Table I. **Genes expressed in rat cortical cell cultures treated with BDNF for 8 h**

Entrez definition	Fold change
Differentiation or proliferation	
Rattus norvegicus dual specificity protein tyrosine phosphatase (rVH6) mRNA	+1.23
Rattus norvegicus SC1 protein mRNA	+1.27
Rattus norvegicus mRNA for CPG2 protein	+10.46
Rattus norvegicus mRNA for urokinase-type plasminogen activator	+2.38
Rattus norvegicus DCN mRNA for decorin	+2.53
Signal transduction	
Rat mRNA for 230-kD phosphatidylinositol 4-kinase, complete cds	+1.71
Rattus norvegicus centaurin alpha mRNA, complete cds	+4.32
Rattus norvegicus nicotinic acetylcholine receptor alpha4-2 mRNA	+2.45
Rattus norvegicus class A calcium channel variant riA-I (BCCA1) mRNA	+7.23
Metabolism or synthesis	
Rattus norvegicus mRNA for ribosomal protein S24	+2.19
Rat lysozyme gene exons 1–4, complete cds	+3.31
Rat PYBP1 mRNA for pyrimidine binding protein 1	+1.85
Rat mRNA for brain acyl-CoA synthetase 2	−0.16
Rat mRNA for ribosomal protein L1	−0.99
Rattus norvegicus palmitoyl protein thioesterase mRNA	−0.89
Rat mRNA for long-chain acyl-CoA synthetase (EC 6.2.1.3)	−0.48
Rat vacuolar protein sorting homolog r-vps33a mRNA, complete cds	−0.13
Rat Rieske iron-sulfur protein mRNA, complete cds	−0.46
Rattus norvegicus ribosomal protein L21 mRNA, complete cds	−0.21
Rat ARSB mRNA for arylsulfatase B, partial cds	−0.49
Endocytosis	
Rattus norvegicus clathrin light chain (LCA1) mRNA	+10.85
Rattus norvegicus vesicular transport protein rvps45 mRNA	−0.53
Other function	
Rattus norvegicus alpha 4 phosphoprotein mRNA	+2.76
Rattus norvegicus cytochrome b558 alpha-subunit mRNA	+7.71
Rattus norvegicus serine proteinase rPC7 precursor (Pcsk7) mRNA, complete cds	+3.72
Rattus norvegicus (Wistar) CaBP1 mRNA	−0.08
Rat CoxVa mRNA for mitochondrial cytochrome c oxidase subunit Va	−0.01
Rattus norvegicus CD59 protein precursor, mRNA, complete cds	−0.12
Rat cytochrome P-450 isozyme 5 (P450 IVB2) mRNA, complete cds	−0.14
Rattus norvegicus mRNA for dual specificity Yak1-related kinase (Dyrk)	−0.32

4-phenyl-1,2,3,6-tetrahydropyridine and 6-hydroxy dopamine (Spina et al., 1992; Frim et al., 1994).The findings above suggest therapeutic potential of NTs for hypoxic-ischemia and various neurodegenerative diseases. However, the beneficial effects of NTs should be compromised with a notion that NTs can exacerbate certain forms of neuronal injury. BDNF, NT-3, or NT-4/5 renders neurons highly vulnerable to deprivation of oxygen and glucose, possibly by enhancing Ca^{2+} influx and nitric oxide production through N-methyl-D-aspartate (NMDA) glutamate receptors (Fernandez-Sanchez and Novelli, 1993; Koh et al., 1995; Samdani et al., 1997). NTs potentiate neuronal death by reactive oxygen species (ROS) or nitric oxide, which was blocked by cycloheximide (Gwag et al., 1995; Park et al., 1998; Kim et al., 1999; Ishikawa et al., 2000). These studies imply that NTs can potentiate necrotic components of neuronal death. In addition, NTs

striatal area. Striatal lesion was analyzed by measuring the injured areas (c), mean ± SEM ($n = 5$ rats per each condition). *Significant difference from the relevant control (saline injected) at $P < 0.05$ using Independent-Samples t test. (C) Phase–contrast (top) and electron (bottom) photomicrographs of cortical neurons 32 h after a sham wash (a and c) or continuous exposure to 100 ng/ml BDNF (b and d). Note that BDNF treatment induces swelling of neuronal cell body (arrow), scattering condensation of nuclear chromatin (arrowhead), and fenestration of plasma membrane characteristic of necrosis. (D) Patterns of BDNF-induced neuronal death were analyzed at 32 h after exposure of cortical cell cultures to 100 ng/ml BDNF. Approximately 200 neurons from control and BDNF-treated cultures were randomly selected and observed under transmission electron microscope. Degenerating neurons were defined as normal, necrosis (see above), or apoptosis (shrinkage of cytoplasm and nuclear membrane rupture with intact plasma membrane). (E) Cortical cell cultures (DIV 12–15) were continuously exposed to 100 ng/ml BDNF, alone or with 100 μg/ml anti-BDNF blocking antibody, 150 nM K252a, 10 μM MK-801, 50 μM CNQX, 10 μM MK 801 plus 50 μM CNQX, 100 μM trolox, or 1 μg/ml cycloheximide (CHX). Neuronal death was analyzed 36 h later by measurement of LDH efflux into the bathing medium, mean ± SEM ($n = 16$ culture wells per condition). *Significant difference from the relevant control (BDNF alone) at $P < 0.05$ using analysis of variance and Student-Neuman-Keuls test.

Figure 2. **BDNF produces ROS in cortical neurons: involvement of protein synthesis.** (A) Cortical cell cultures (DIV 12–15) were exposed to a sham wash (●) or 100 ng/ml BDNF (○). Levels of ROS in neurons were analyzed at indicated times by measuring fluorescence intensity of oxidized DCDHF-DA (DCF), mean ± SEM (n = 30–35 neurons randomly chosen from four culture wells per condition). *Significant difference from the relevant control (sham wash) at $P < 0.05$ using analysis of variance and Student-Neuman-Keuls test. (B and C) Fluorescence photomicrographs (B) and quantitation (C) of DCF in cortical neurons after 32 h exposure of cortical cell cultures (DIV 12–15) to a sham wash (CTRL) or 100 ng/ml BDNF, alone (BDNF) or in the presence of 1 μg/ml cycloheximide (CHX) or 100 μM trolox (TROLOX). *Significant difference from the control (CTRL); [#]significant difference from the BDNF control (BDNF alone) at $P < 0.05$ using analysis of variance and Student-Neuman-Keuls test.
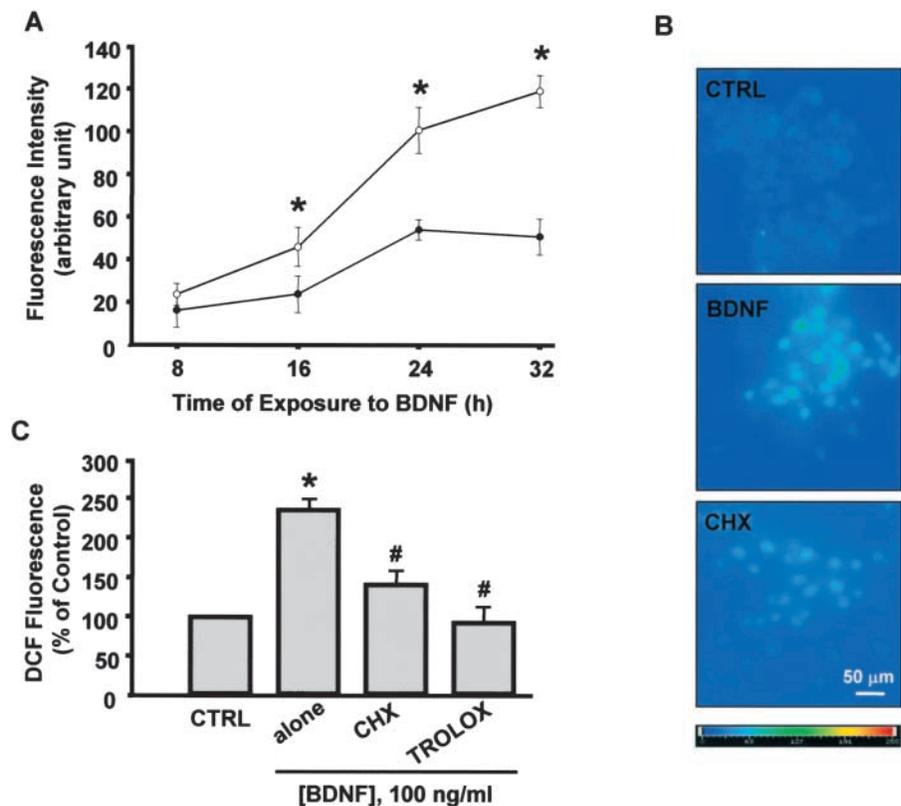
can directly induce death of neurons, oligodendrocytes, Schwann cells, and tumor cells (Casaccia-Bonnefil et al., 1996; Muragaki et al., 1997; Sedel et al., 1999; Soilu-Hanninen et al., 1999; Giehl et al., 2001).

Recently, we have found that cortical cell cultures exposed to NTs can undergo neuronal death. The present study was performed to determine the morphological patterns, apoptosis versus necrosis, of neuronal death induced by NTs. In addition, we set out experiments to examine the possibility that necrotic pathways (e.g., excitotoxicity and oxidative stress) and putative target genes would mediate the neurotoxic action of NTs.

Results

BDNF induces neuronal cell necrosis

Administration of NTs did not produce neuronal injury in cortical cell cultures within 1 d when NTs can prevent neuronal apoptosis and potentiate neuronal death after deprivation of oxygen and glucose (Koh et al., 1995). However, wide spread neuronal death occurred in cortical cell cultures continuously exposed to various concentrations (10, 30, or 100 ng/ml) of BDNF or NT-3 for 36–48 h (Fig. 1 A). Near complete neuronal death was observed within 48 h after exposure to BDNF or NT-3. Neuronal death was not induced by exposure to NGF, since cortical neurons express TrkB and TrkC but not TrkA (Knüsel et al., 1992; Widmer et al., 1992). The neurotoxic effects of NTs were observed in striatal areas 2 d after the intrastriatal injections of BDNF in adult rat brain (Fig. 1 B). The cell body swelling preceded the latent neuronal death as shown in the process of necrotic neuronal death after insults to excitotoxins or

prooxidants (Gwag et al., 1997; Ryu et al., 1999). The ultrastructural analysis of degenerating neurons in BDNF-treated cortical cultures reveals swelling of cytoplasmic organelles, earlier collapse of plasma membrane than nuclear membrane, and scattering condensation of nuclear chromatin (Fig. 1, C and D).

BDNF-induced neuronal necrosis was completely blocked by inclusion of K252a, an inhibitor of the Trk receptor tyrosine kinases, and 100 μg/ml anti-BDNF blocking antibody, suggesting that Trk mediates the neurotoxic actions of NTs (Fig. 1 E). Since excess activation of ionotropic glutamate receptors sensitive to NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainite and oxidative stress cause neuronal death exclusively through necrosis (Gwag et al., 1997; Ryu et al., 1999), experiments were performed to examine if glutamate receptor antagonists or antioxidants would block BDNF-induced neuronal cell necrosis. Neither the NMDA receptor antagonist MK-801 nor the AMPA/kainite receptor antagonist CNQX reduced BDNF-induced neuronal necrosis, suggesting that excitotoxicity does not mediate BDNF neurotoxicity (Fig. 1 E). However, concurrent administration of trolox, a lipophilic analogue of vitamin E, completely blocked BDNF neurotoxicity. Interestingly, BDNF-induced neuronal cell necrosis was also blocked by addition of cycloheximide, a protein synthesis inhibitor. Thus, BDNF appears to produce free radical–mediated neuronal cell necrosis in a protein synthesis-dependent manner.

BDNF produces ROS in cortical neurons

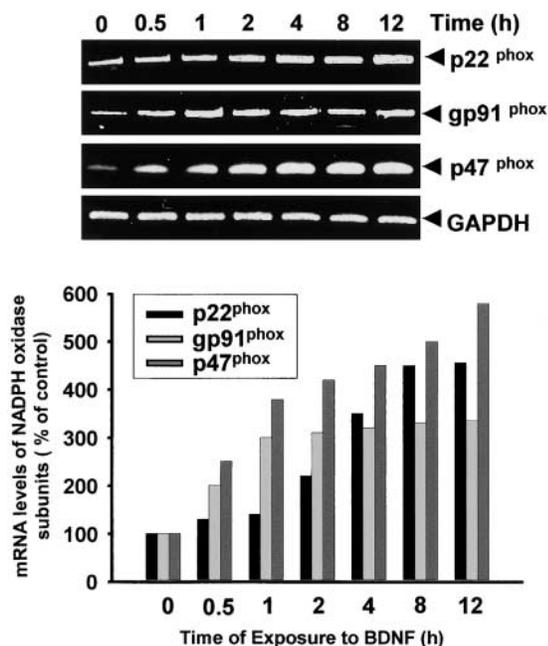Additional experiments were performed to examine whether BDNF would produce ROS in cortical cell cultures. The

Figure 3. **BDNF increases mRNA levels of NADPH oxidase subunits in cortical cultures.** RT-PCR analysis of NADPH oxidase subunits (p22-phox, gp91-phox, and p47-phox) and GAPDH mRNA expression in cortical cell cultures (DIV 12) exposed to 100 ng/ml BDNF for indicated times. The mRNA level of each NADPH oxidase subunit was normalized to the relevant control value ($t = 0$). Similar results were observed from three separate experiments.

overall level of ROS was determined by analyzing oxidation of 2′,7′-dichlorodihydrofluorescein (DCDHF) to dichlorofluorescein (DCF). The fluorescent intensity of DCF was increased in cortical neurons exposed to BDNF for 16 h (Fig. 2 A). The intraneuronal levels of ROS ($[ROS]_i$) were further increased over 24–32 h. Treatment with BDNF did not increase levels of ROS in astrocytes that grew as a monolayer underneath neurons (unpublished data). The BDNF-induced production of $[ROS]_i$ was prevented by concurrent addition of cycloheximide and trolox (Fig. 2 B, and C). Thus, BDNF likely produces ROS presumably through synthesis of prooxidant proteins.

BDNF increases expression of NADPH oxidase

We used cDNA microarray assay to screen target genes for the prooxidant action of BDNF in cortical cell cultures. The microarray analysis revealed that various genes were regulated in cortical cell cultures exposed to BDNF for 8 h (Table I). The target genes of BDNF mostly play a role in differentiation, endocytosis, metabolism, and signal transduction that likely reflect neurotrophic actions of NTs. Among the BDNF-sensitive genes, cytochrome b_{558} was chosen as a candidate gene for the neurotoxic actions of the NT, since it constitutes p22-phox and gp91-phox subunits of NADPH oxidase, a prooxidant enzyme generating superoxide from oxygen. RT-PCR analysis showed an increase in mRNA levels of p22-phox and gp91-phox within 2 h after treatment with BDNF (Fig. 3). Levels of both mRNAs were maximally increased 4 h later, which lasted over the next 12 h. The mRNA levels of p47-phox subunit were also increased gradually from 30 min after
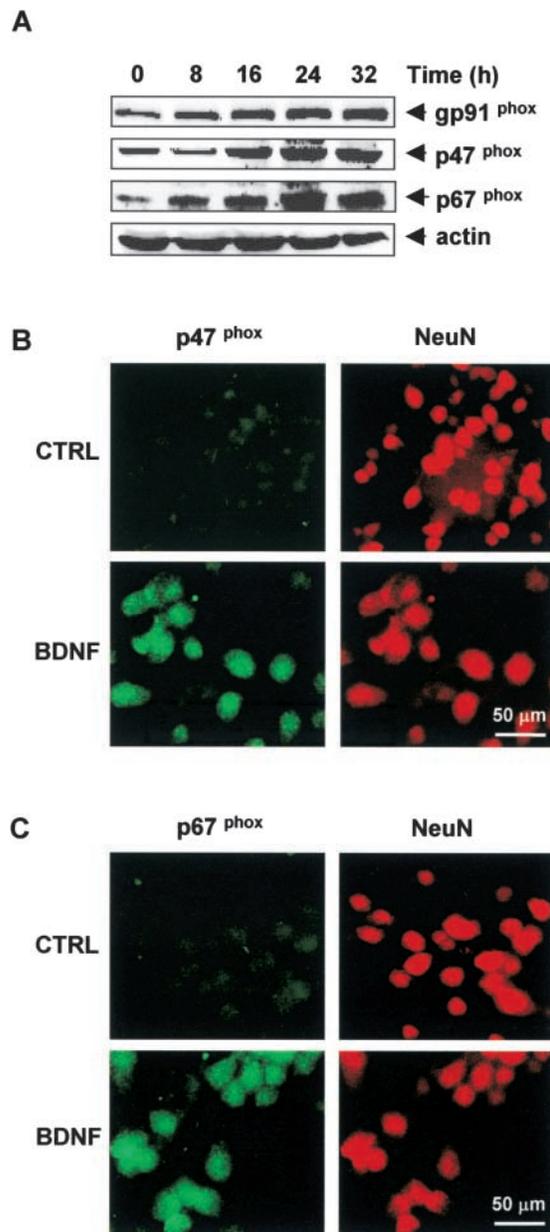

Figure 4. **BDNF increases expression of NADPH oxidase subunits in cortical neurons.** (A) Western blot analysis of NADPH oxidase subunits (gp91-phox, p47-phox, and p67-phox) and actin in cortical cell cultures after exposure to 100 ng/ml BDNF for indicated times. Similar results were obtained from three separate experiments. (B) Fluorescence photomicrograph of cortical cell cultures immunolabeled with NeuN (right; anti–mouse immunoglobulin G conjugated with Texas red) and anti-p47-phox (left; anti–goat immunoglobulin G conjugated with FITC) after exposure to a sham wash (top) and 100 ng/ml BDNF (bottom) for 32 h. (C) Same as B except that cultures were immunolabeled with anti–p67-phox antibody (anti–goat immunoglobulin G conjugated with FITC) instead of anti–p47-phox. Note that levels of p47-phox and p67-phox were increased selectively in neurons after exposure of mixed cortical cell cultures to BDNF.

administration of BDNF. Western blot experiments were performed to analyze protein levels of NADPH oxidase subunits using available antibodies for gp91-phox, p47-phox, and p67-phox. Expression of these subunits was increased over 16–32 h in cortical cell cultures exposed to BDNF (Fig. 4 A). Immu-
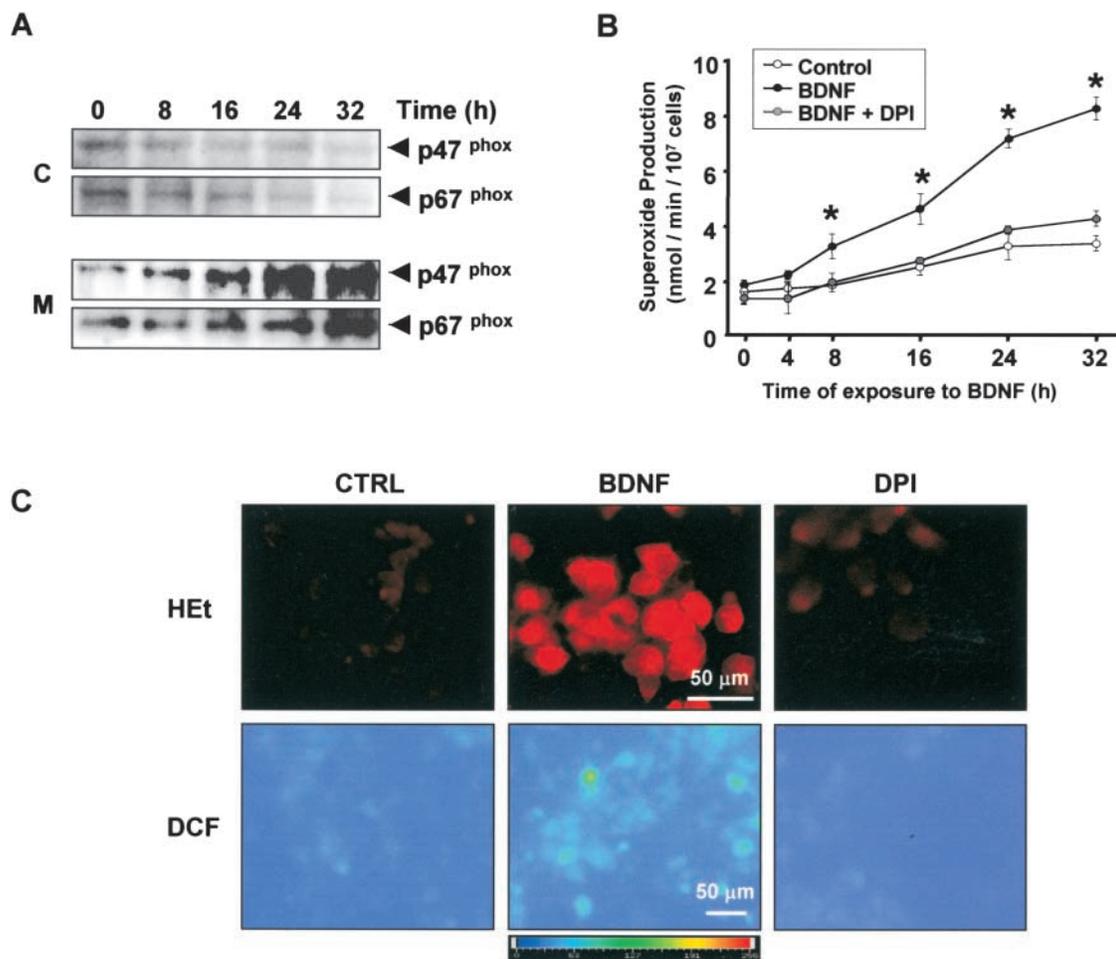
Figure 5. **Translocation and activation of NADPH oxidase by BDNF.** (A) Western blot analysis of the cytosolic fraction (C) and the membrane fraction (M) using anti–p47-phox and anti–p67-phox antibodies that were obtained from cortical cell cultures (DIV 12) after exposure to 100 ng/ml BDNF for the indicated times. (B) Superoxide production was analyzed by measuring reduction of cytochrome c in cortical cultures exposed to a sham wash (○) or 100 ng/ml BDNF with (gray colored circles) or without 3 nM DPI (●) for indicated times, mean ± SEM (n = 12 culture wells per condition). *Significant difference from the sham control at $P < 0.05$ using analysis of variance and Student-Neuman-Keuls test. (C) Fluorescence photomicrograph of the oxidized hydroethydine (HEt, top) and DCF (bottom) in cortical neurons after exposure to a sham operation (CTRL), 100 ng/ml BDNF (BDNF), or 100 ng/ml BDNF plus 3 nM DPI (DPI) for 32 h.

nocytochemistry was performed to identify which types of cells express NADPH oxidase in cortical cell cultures containing neurons and glia. Immunoreactivity to p47-phox or p67-phox antibody was slightly observed in cortical neurons but not in astrocytes after a sham operation. Signals of p47-phox and p67-phox were increased exclusively in neurons 32 h after exposure of cortical cell cultures to BDNF (Fig. 4, B and C).

BDNF produces ROS through activation of NADPH oxidase

Activation of NADPH oxidase involves translocation of the cytosolic p47-phox and p67-phox subunits into the plasma membrane (Clark et al., 1989). The levels of p47-phox and p67-phox were reduced in the cytosolic fraction and increased in the membrane fraction from cortical cell cultures exposed to BDNF for 16–32 h (Fig. 5 A), suggesting that treatment with BDNF results in activation of NADPH oxidase. This was further supported by increased production of superoxide up to three- to fourfold in cortical cell cultures treated with BDNF for 16–32 h as determined by the reduction of cytochrome c

(Fig. 5 B). Treatment with BDNF resulted in the increased oxidation of hydroethydine and 2′, 7′-dichlorodihydrofluorescein to ethidium and dichlorofluorescein, respectively, exclusively in neurons (Fig. 5 C). We analyzed the inhibitory effects of diphenylene iodonium (DPI), a selective inhibitor of flavoprotein-dependent enzymes such as cytochrome b_{558}, the plasma membrane–spanning subunit of NADPH oxidase (O'Donnell et al., 1993), against BDNF-induced superoxide production. DPI completely blocked BDNF-induced superoxide production (Fig. 5, B and C). Thus, BDNF produces oxidative stress in cortical neurons through NADPH oxidase-mediated production of superoxide.

Activation of NADPH oxidase mediates BDNF neurotoxicity

We examined if activation of NADPH oxidase would contribute to BDNF-induced neuronal death. Coadministration of NADPH oxidase inhibitors, 3–10 nM DPI or 10–30 μM 4-(2-aminoethyl)-benzensulfonyl fluoride (AEBSF), significantly reduced swelling of neuronal cell body and
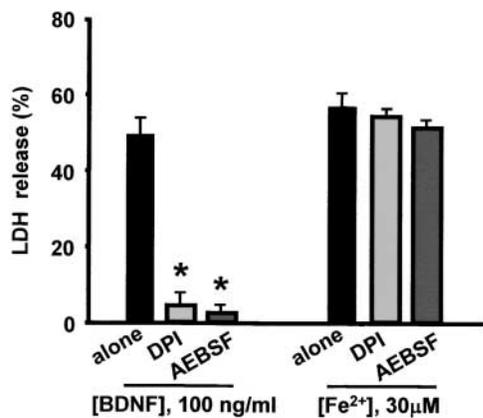
Figure 6. **Activation of NADPH oxidase mediates BDNF-induced neuronal death.** Cortical cell cultures (DIV 12–15) were exposed to 100 ng/ml BDNF or 30 μM Fe²⁺, alone or in the presence of 3 nM DPI or 10 μM AEBSF. Neuronal death was analyzed 36 h later by measurement of LDH efflux into the bathing medium, mean ± SEM (*n* = 16 culture wells per condition). *Significant difference from the relevant control (BDNF or Fe²⁺ alone) at P < 0.05 by Student-Neuman-Keuls test.

Figure 7. **DPI or trolox enhances the neuroprotective effect of BDNF against serum deprivation.** Neuron-rich cortical cell cultures (DIV 7) were deprived of serum, alone (serum deprivation) or in the presence of 100 ng/ml BDNF, 100 ng/ml BDNF plus 3 nM DPI, 3 nM DPI, 100 ng/ml BDNF plus 100 μM trolox, or 100 μM trolox. Neuronal death was assessed 24 and 48 h later by counting viable neurons, mean ± SEM (*n* = 16 fields randomly chosen from four culture wells per condition). *Significant difference from the relevant control (serum deprivation alone) at P < 0.05 using analysis of variance and Student-Neuman-Keuls test.

neuronal death 36 h after exposure of cortical cell cultures to BDNF (Fig. 6). However, neither DPI nor AEBSF prevented oxidative neuronal death evolving 24 h after exposure of cortical cell cultures to 30 μM Fe²⁺ (Fig. 6), which was shown to produce hydroxyl radical through Fenton reaction and neuronal cell necrosis in cortical cell cultures (Ryu et al., 1999).

Finally, the putative role of NADPH oxidase was studied regarding the antiapoptosis action of NTs. As reported previously (Koh et al., 1995), administration of BDNF prevented apoptosis of cortical neurons deprived of serum for 24 h (Fig. 7). Neither DPI nor trolox alone reduced serum deprivation-induced neuronal apoptosis. The antiapoptotic action of BDNF was insensitive to inclusion of DPI or trolox. Interestingly, the protective effects of BDNF disappeared within 48 h after serum deprivation. The delayed neuronal death evolving in the presence of BDNF was accompanied by the marked swelling of cell bodies (unpublished data) and attenuated by concurrent addition of DPI or trolox (Fig. 7). The results suggest that expression and activation of NADPH oxidase mediate the pronecrosis, but not antiapoptosis, action of BDNF.

Discussion

We report that prolonged exposure to BDNF produces neuronal cell necrosis in cortical cell cultures. Oxidative stress and protein synthesis are required for BDNF neurotoxicity. DNA microarray revealed transcriptional activation of NADPH oxidase in BDNF-treated cortical cell cultures. Increased expression and activation of NADPH oxidase mediate oxidative stress and neuronal death by BDNF.

Apoptosis and necrosis comprise two major patterns of neuronal death occurring under physiological and pathological conditions. Apoptosis or shrinkage necrosis is characterized by early collapse of nuclear membrane and aggregated condensation of nuclear chromatin evident under electron
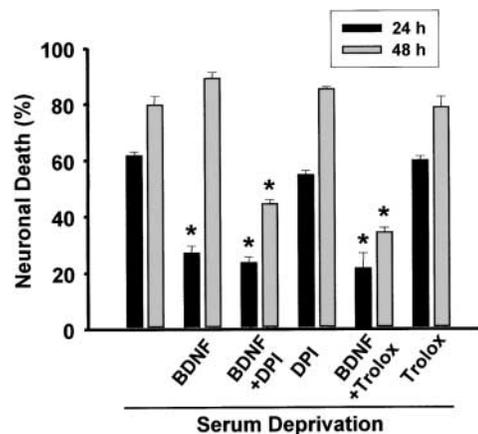
microscope (Kerr, 1972). Necrosis denotes swelling necrosis that is accompanied by early collapse of plasma membrane and scattering condensation of nuclear chromatin (Wyllie et al., 1980). According to the original criteria above, prolonged exposure to BDNF produced swelling necrosis in cortical cell cultures. This supports a hypothesis that NTs can act as pronecrotic factors while they promote neuronal survival by preventing apoptosis (Gwag et al., 1995; Koh et al., 1995). The Trk receptors appear to mediate the pronecrotic effects of BDNF in cortical cell cultures that express Trk B and Trk C but not the p75 NT receptor (p75NTR) (Springer et al., 1990; Koh et al., 1995).

Excitotoxicity and oxidative stress have been recognized as routes to neuronal cell necrosis in the nervous system. Excess activation of NMDA, AMPA, or kainate receptors cause influx of Ca²⁺, Na⁺, Cl⁻ and H₂O that results in rapid swelling of cell body, collapse of plasma membrane, and scattering condensation of nuclear chromatin (Hajos et al., 1986; Choi, 1987; Dessi et al., 1993; Gwag et al., 1997). The similar morphological patterns were observed in cortical neurons exposed to prooxidants such as Fe²⁺ or Zn²⁺ (Gwag et al., 1995; Park et al., 1998; Kim et al., 1999). Although none of the ionotropic glutamate receptor antagonists prevented BDNF-induced neuronal cell necrosis, the membrane-permeable form of vitamin E completely blocked the BDNF neurotoxicity. This implies that oxidative stress mediates the pronecrotic action of BDNF. In support of this, treatment with BDNF produced ROS before swelling of neuronal cell body.

It has been well documented that administration of NTs increases neuronal survival and differentiation in vitro. It is of notice that the neurotrophic effects of NTs have been examined primarily in defined media containing putrescin and selenium as antioxidants (Alderson et al., 1990; Friedman et al., 1993; Cohen et al., 1994). Thus, oxidative stress and

neuronal death by prolonged exposure to NTs appear to be masked, exclusively revealing roles of NTs as survival and differentiation factors. We have studied effects of NTs in mixed cortical cultures of neurons and glia in culture media without serum or antioxidants. Since neurons growing on astrocytes can survive over several days in the absence of serum or antioxidants, this culture condition has been widely used to study mechanisms of excitotoxicity, oxidative stress, and apoptosis. Under this culture condition, prolonged exposure to NTs induced degeneration of cortical neurons that were not observed in the presence of serum or antioxidants (unpublished data). This suggests that neurontrophins act as neurotrophic or neurotoxic factors depending on the presence of antioxidants.

Evidence is being accumulated that NTs potentiate neuronal death after deprivation of oxygen and glucose or administration of NMDA, free radical-inducing agents (Fe^{2+} or buthionine sulfoximine), β amyloid, or nitric oxide in vitro (Yankner et al., 1990; Gwag et al., 1995; Koh et al., 1995; Ishikawa et al., 2000). The potentiation effects of NTs have been reported in adult rat brain subjected to administration of Fe^{2+} or cerebral ischemia (Won et al., 2000; Bates et al., 2002). The present findings that NTs directly cause oxidative stress likely underlie the potentiation effects of NTs on some neuronal injuries.

Since concurrent treatment with cycloheximide inhibited ROS production and neuronal death after exposure to BDNF, we reasoned that expression of ROS-regulating enzymes would be altered in cortical neurons exposed to BDNF. We first examined mRNA expression of antioxidant enzymes such as manganese-superoxide dismutase and glutathione peroxidase. RT-PCR analysis showed that mRNA levels of these enzymes were increased up to two- to three-fold 24 h after exposure of cortical cell cultures to BDNF (Kim and Gwag, unpublished data). The late expression of the antioxidant enzymes appears to represent adaptive responses to oxidative stress that is evolved within 16 h after administration of BDNF. We then performed DNA microarray analysis to search for candidate molecules involved in the prooxidant effects of BDNF and found that mRNA levels of the p22-phox and gp91-phox NADPH oxidase subunits were increased within 8 h in cortical cell cultures exposed to BDNF or NT-3 and NT-4/5.

NADPH oxidase was first discovered in phagocytes as a superoxide-producing enzyme via one-electron reduction of oxygen (Patriarca et al., 1971; Prough and Masters, 1973). NADPH oxidase consists of cytochrome b_{558}, a membrane heterodimer of gp91-phox and p22-phox, and three cytosolic subunits, p40-phox, p47-phox, and p67-phox (Parkos et al., 1987; Lomax et al., 1989; Okamura et al., 1990). Activation of NADPH oxidase is initiated by membrane translocation of p47-phox and p67-phox to interact with cytochrome b_{558} (Clark et al., 1989; Heyworth et al., 1991). Treatment with BDNF increased mRNA (p22-phox, p47-phox, gp91-phox) and protein (p47-phox, p67-phox, gp91-phox) levels of NADPH oxidase subunits selectively in cortical neurons. Increased expression of subunits was followed by membrane translocation of p47-phox and p67-phox and subsequent production of superoxide presumably in the cytosol.

The weak oxidant superoxide can be converted to more reactive oxidants including hydrogen peroxide, hydroxyl radical, and other ROS primarily in mitochondria (Bannister et al., 1982; Miller and Britigan, 1995). BDNF-treated cortical neurons revealed mitochondrial ROS production within 16–24 h as determined by Mitotracker red CM-H$_2$Xros (unpublished data). The mitochondrial ROS production and the overall oxidative stress by BDNF were reduced in the presence of the selective inhibitors of NADPH oxidase. This implies that BDNF-induced superoxide results in the secondary ROS production through mitochondria and the delayed neuronal death.

Although activation of NADPH oxidase is required for the oxidative neuronal necrosis by BDNF, it does not mediate the antiapoptosis action of the NT. Surprisingly, the slowly evolving oxidative stress overrides the neuroprotective effect of BDNF against serum deprivation-induced apoptosis. Thus, the neurotrophic effect of BDNF is enhanced with blockade of oxidative stress by NADPH inhibitors or antioxidants.

Several lines of evidence suggest that NADPH oxidase participates in the process of neuronal injury under pathological conditions. The infarct size after transient middle cerebral artery occlusion was significantly reduced in mutant mice with defect in NADPH oxidase (Walder et al., 1997). Membrane translocation of p47-phox and p67-phox was observed in Alzheimer's disease brain (Shimohama et al., 2000). Although mechanisms underlying activation of NADPH oxidase remain to be delineated, Zn^{2+} can mediate activation of NADPH oxidase in neurons and astrocytes. Zn^{2+} is released from the presynaptic terminal of glutamatergic neurons in an activity-dependent manner and enters into adjacent neurons primarily through voltage-gated Ca^{2+} channels and Ca^{2+}-permeable glutamate receptors (Choi and Koh, 1998). Accumulation of Zn^{2+} causes ROS-mediated neuronal necrosis in part through protein kinase C–dependent activation of NADPH oxidase (Kim et al., 1999; Noh and Koh, 2000). Fibrillary forms of β amyloid activate NADPH oxidase in microglial cells (Bianca et al., 1999), which can contribute to ROS production and degeneration of adjacent neurons. In addition to the endogenous neurotoxins Zn^{2+} and β amyloid, NTs transcriptionally and translationally activate NADPH oxidase in neurons and can act as another neurotoxic substance in the nervous system.

We report for the first time that NTs can act as a prooxidant through activation of NADPH oxidase and cause neuronal cell necrosis through production of ROS. Although NTs reveal neurotrophic activity by preventing apoptosis and promoting regeneration (Levi-Montalcini, 1987; Barde, 1994; Deshmukh and Johnson, 1997), the current findings suggest that the neurotrophic action of NTs should be exploited with blockade of the pronecrotic effects.

Materials and methods

Primary cortical cell culture

Rat cortical cell cultures were prepared from the 17-d-old fetal brain, and the neocortices were mechanically triturated as described previously (Noh and Gwag, 1997). Dissociated cells were plated on 6-well plates and 24-well plates (approximately three cortices per plate) or on glass bottom 35-mm dishes for ROS imaging. Plating media consist of Eagle's minimal essential medium (MEM, Earle's salts, supplied glutamine-free) supple-

mented with 5% horse serum, 5% FBS, 21 mM glucose, 26.5 mM bicarbonate, and 2 mM L-glutamine. For neuron–glia mixed cultures, 10 μM cytosine arabinoside (Ara C) was included to stop the overgrowth of nonneuronal cells to cultures at days in vitro (DIV) 5–7 when glial cells were confluent underneath neurons. After 2 d, cultures were then fed with plating medium lacking fetal serum twice a week. Cultures were maintained at 37°C in a humidified 5% CO_2 incubator. For neuron-rich cultures (>95%), 2.5 μM Ara C was included to cultures at 2–3 d in vitro (DIV 2–3) as described previously (Gwag et al., 1997).

Induction and analysis of cell death

Mixed cortical cell cultures (DIV 12–14) were rinsed in serum-free MS (MEM supplemented with 26.5 mM sodium bicarbonate and 21 mM glucose) and then exposed to various concentrations of NGF, BDNF, or NT-3 in serum-free MS. Neuronal cell death was analyzed by measuring the level of lactate dehydrogenase (LDH) released into the bathing medium. The percentage of neuronal death was normalized to the mean LDH value released after a sham control (defined as 0%) or continuous exposure to 500 μM NMDA for 24 h (defined as 100%) The latter produces complete neuronal death within 24 h. For experiments for serum deprivation, neuron-rich cortical cell cultures (DIV 7) were placed into serum-free MS containing 1 μM MK-801 as described (Gwag et al., 1995). Neuronal death was analyzed 24 and 48 h later by counting viable neurons excluding Trypan blue stained.

Transmission electron microscopic observation

Cultures were fixed in Karnovsky's fixative solution (1% paraformaldehyde, 2% glutaraldehyde, 2 mM calcium chloride, 100 mM cacodylate buffer, pH 7.4) for 2 h, washed with cacodylate buffer, and postfixed in 1% osmium tetroxide and 1.5% potassium ferrocyanide for 1 h. Cells were then stained en bloc in 0.5% uranyl acetate, dehydrated through graded ethanol series, and embedded in Poly/Bed 812 resin. Cells were sectioned using Reichert Jung Ultracut S (Leica). After staining cells with uranyl acetate and lead citrate, cells were observed and photographed under ZEISS EM 902A.

Intrastriatal injection of BDNF in rat brain

Adult male Sprague-Dawley rats weighing 250–300 g were anesthetized intraperitoneally with chloral hydrate (400 mg/ml). Animals were placed in a Kopf stereotaxic apparatus and injected with 1 μg/μl of BDNF (dissolved in 0.9% NaCl [saline]) or saline alone in the striatum at the following coordinates: 1.0 mm rostral to bregma, 3.0 mm lateral to the midline, and 5.0 mm ventral from the dural surface. For each injection, a volume of 5 μl was delivered for 10 min via a 10-μl Hamilton syringe. 3 min were allowed before syringe withdrawal and wound closure. These rats were killed 2 d later. Animals were anaesthetized and then perfused transcardially with PBS followed by 3% paraformaldehyde. The brains were immediately removed, postfixed, and then sectioned (8 μm) on a microtome (TPI, Inc.). Sections including the injection site were collected and stained with hematoxylin and eosin. The lesion area was analyzed as described previously (Won et al., 2000). Six serial sections including the needle track and the largest injury area evident by decrease in staining intensity were included for analysis of injury per each animal. The striatal section stained with hematoxylin and eosin was scanned analyzed using a computer-assisted image analysis system (SigmaScan, CA/TINA 2.0; KAIST).

ROS imaging

Cortical cell cultures (DIV 12–15) grown on glass bottom dishes were loaded with 10 μM dichlorodihydro fluorescein diacetate (DCDHF-DA) or 5 μM hydroethidium (Molecular Probes) plus 2% Pluronic F-127 in Hepes-buffered control salt solution (HCSS) buffer containing (in mM): 120 NaCl, 5 KCl, 1.6 MgCl$_2$, 2.3 CaCl$_2$, 15 glucose, 20 Hepes, and 10 NaOH. Cultures were incubated for 20 min at 37°C and washed three times with HCSS buffer. The fluorescence signal of oxidized DCDHF was observed at room temperature on the stage of a Nikon Diaphot inverted microscope equipped with a 100 W xenon lamp and filter (for oxidized DCDHF, excitation = 488 nm and emission = 510 nm; for hydroethidine, excitation = 546 nm and emission = 590 nm). The fluorescence images were analyzed using a QuantiCell 700 system (Applied Imaging).

RNA preparation and cDNA microarray analysis

Total RNA was isolated from cortical cell cultures (DIV 12) by using RNA zol B (Tel-Test Inc.). Approximately 1 μg of total RNA was used to synthesize cDNA labeled with [α-^{33}p] dATP that was hybridized to rat gene filter membranes (Research Genetics) at 42°C for 12–18 h. The membranes were washed in 2× SSC buffer and 1% SDS at 50°C for 20 min, 0.5× SSC and 1% SDS at room temperature for 15 min, and then wrapped up in

plastic wrap and apposed to a phosphorimager cassette. After exposure of gene filters, the hybridization pattern was analyzed using Pathways™ 4-universal microarray analysis software (Invitrogen).

RT-PCR

RT-PCR experiments were performed to confirm the target genes of BDNF derived from cDNA expression microarray. Total RNA (1 μg each) was incubated in a reaction mixture containing dNTP (2.5 mM each), RNasin (0.5 U), oligo dT primer (100 ng), and MMLV reverse transcriptase (200 U) at 37°C for 1 h. The samples were incubated at 92°C for 10 min and transferred to 4°C. The reverse transcribed cDNA was subjected to PCR amplification. PCR was performed according to manufacturer's procedure (Takara Shuzo Co.) sequentially (denaturation-annealing-extension) at the following conditions: for p47-phox, 94°C for 30 S, 55°C for 30 S, and 72°C for 60 S (28 cycles); for p22-phox (homologous to cytochrome b$_{558}$ in microarray) and gp91-phox, 94°C for 45 S, 60°C for 60 S, and 72°C for 120 S (33 cycles); and for GAPDH, 94°C for 35 S, 55°C for 45 S, and 72°C for 90 S (25 cycles). Primer sequences used were as follows (5′-3′): for p22-phox, GAATTCCGATGGGGCAGATCGAGTGGGCCA (forward) and GGATCCCGTCACACGACCTCATCTGTCACT (reverse); for p47-phox, CAGCCAGCACTATGTGTACA (forward) and GAACTCGTAGATCTCGGTGAA (reverse); for gp91-phox, GAATTCCGATGGGGAACTGGGCTGTGAATG (forward) and GGATCCCGTTAGAAGTTTTCCTTGTTGAAA (reverse); for GAPDH, TCCATGACAACTTTGGCATCGTGG (forward) and GTTGCTGTTGAAGTCACAGGAGAC (reverse). PCR products were run on a 1.2% agarose gel and visualized with ethidium bromide. The relative amount of mRNA was measured using LAS-1000 systems (Fuji Photofilm Co.), normalized to levels of GAPDH mRNA. DNA sequencing was performed with Big Dye Terminator Chemistry from PerkinElmer on ABI PRISM™ 377 DNA sequencer.

Western blot analysis

Cortical cell cultures were lysed in a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 1% Nonidet P-40, 150 mM NaCl, 0.5% deoxycholic acid, 0.1% SDS, 1 mM PMSF, 10 μg/ml pepstain A, and 100 μg/ml leupeptin. Cell lysates were centrifuged at 12,000 g for 10 min ∼25 μg of protein was subjected to electrophoresis on 12% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane. The blot was incubated in 2.5% BSA for 1 h, incubated with goat polyclonal primary antibodies, anti–gp91-phox, anti–p67-phox, or anti–p47-phox antibodies (1:1,000, Santa Cruz Biotechnology, Inc.), and then reacted with a biotinylated anti–goat secondary antibody. Immunoreactivity was detected with Vectastain ABC kit (Vector Laboratories) and luminol for ECL (Intron). The signal was analyzed by quantitative densitometry using LAS-1000 systems (Fuji Photofilm Co.).

Subcellular fractionation

Cortical cell cultures were washed with ice-cold PBS and resuspended in an isotonic buffer containing 10 mM Hepes, pH 8.0, 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 2 mM PMSF, 100 μg/ml leupeptin, and 10 μg/ml pepstatin A. For isolating the cytosol and membrane fraction, the lysate was homogenized with a homogenizer (KONTE), centrifuged at 9,000 g for 10 min, and the supernatant was then centrifuged at 100,000 g for 1 h. The membrane fraction was obtained by resuspending the pellet with 50 μl lysis buffer, and the cytosolic fraction was obtained from the supernatant.

Immunocytochemistry

Cortical cell cultures (DIV 12–14) grown on glass bottom dishes were fixed in 4% paraformaldehyde for 30 min, incubated in 10% horse serum for 1 h, and double immunolabeled with a mouse monoclonal antibody against NeuN (1:400 dilution; Chemicon) and a goat polyclonal antibody against p47-phox or p67-phox (1:200 dilution; Santa Cruz Biotechnology Inc.) for 2–4 h. Cultures were then reacted with fluorescein isothiocyanate-conjugated anti–goat IgG (1:200 dilution; Organon Teknika Corp.) and Texas red–conjugated anti–mouse IgG (1:200; Vector Laboratories) for 1–2 h. The fluorescence images were collected and analyzed with a fluorescence microscopy (ZEISS) equipped with the Real-14™ precision digital camera (Apogee Instrument) and ImagePro Plus Plug-in.

Measurement of NADPH oxidase activity

Superoxide production was measured in a quantitative kinetic assay based on the reduction of cytochrome c (Mayo and Curnutte, 1990). Cortical cell cultures were suspended in PBS and incubated in a reaction mixture containing 0.9 mM CaCl$_2$, 0.5 mM MgCl$_2$, and 7.5 mM glucose, 75 μM cytochrome c (Sigma-Aldrich), and 60 μg/ml super oxide dismutase (Sigma-Aldrich) for 3 min at 37°C. The superoxide production was determined by

measuring the absorbance of cytochrome c at 550 nm using a Thermomax microplate reader and associated SOFTMAX Version 2.02 software (Molecular Devices Corp.).

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