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ETHANOL INHIBITS BRAIN-DERIVED NEUROTROPHIC FACTOR-MEDIATED INTRACELLULAR SIGNALING AND ACTIVATOR PROTEIN-1 ACTIVATION IN CEREBELLAR GRANULE NEURONS

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Abstract—Developmental exposure to ethanol causes profound damage to the cerebellum, ranging from aberration in neuronal differentiation to cell loss. As a major neurotrophic factor, brain-derived neurotrophic factor (BDNF) and its receptor TrkB are expressed in the developing, as well as adult, cerebellum. Many neurotrophic effects of BDNF are mediated by gene transcription. We hypothesized that ethanol interfered with BDNF signaling and disrupted BDNF-regulated transcriptional activity. Using a transgenic mouse model expressing an activator protein-1 (AP-1) luciferase reporter construct, we demonstrated that BDNF stimulated AP-1 transactivation in cultured cerebellar granule neurons. This observation was validated by the study using a human neuronal cell line expressing inducible TrkB (TB8 neuroblastoma cells). BDNF induced AP-1 transactivation, as well as increased the binding activity of AP-1 protein complex to a DNA sequence containing AP-1 sites in TB8 cells. BDNF-mediated AP-1 activation was mediated by PI3K/Akt and JNK pathways; BDNF activated Akt and JNKs, and blocking these pathways significantly inhibited BDNF-stimulated AP-1 transactivation. More importantly, ethanol inhibited BDNF-mediated activation of PI3K/Akt and JNKs, and blocked BDNF-stimulated AP-1 activation. Since ethanol did not affect either the expression or autophosphorylation of TrkB, it could be concluded that the site of ethanol action was downstream of TrkB. The present study establishes that this AP-1 reporter transgenic mouse model is valuable for assessing AP-1 activity in the CNS neurons. Our results provide an insight into molecular mechanism(s) of ethanol action.

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Abbreviations: AP-1, activator protein-1; BDNF, brain-derived neurotrophic factor; CGN, cerebellar granule neuron; DTT, [dl]-dithiothreitol; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; NT, neurotrophin; PI3K, phosphatidylinositol 3-kinase; PMSF, phenylmethylsulfonyl fluoride; Tet, tetracycline; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TPBS, 0.010 M phosphate-buffered saline (pH 7.4) and 0.10% Tween-20; TRE, 12-*O*-tetradecanoylphorbol-13-acetate-responsive element.

Key words: apoptosis, development, fetal alcohol syndrome, neurotrophins, signal transduction, transcription factor.

Ethanol exposure during development causes dysfunction of the CNS (Goodlett and Horn, 2001). The cerebellum is one of CNS regions that are particularly vulnerable to ethanol toxicity (Marcus, 1987; Eckardt et al., 1998; Guerri, 1998). Ethanol-induced damage includes a decrease in measures of cerebellar size (the weight of cerebellum, DNA/protein contents, the growth of cerebellar volume and circumference of the vermis; Nathaniel et al., 1986; Miller, 1996; Maier et al., 1997, 1999), alteration in neurite growth (Bearer et al., 1999; Zou et al., 1993), abnormality in synaptic development (Mohamed et al., 1987; Lolov et al., 1988; Dlugos and Pentney, 1997) and cell loss; exposure to ethanol during development causes a well-characterized decrease in the number of both cerebellar granule neurons and Purkinje neurons (Borges and Lewis, 1983a,b; Bonthius and West 1990; Hamre and West, 1993; Napper and West, 1995; Maier et al., 1999). However, the molecular mechanisms underlying ethanol-induced injuries to the developing cerebellum remain incompletely elucidated.

The mammalian neurotrophin family includes nerve growth factor, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and NT-4/5 (Lessmann et al., 2003). These growth factors were originally identified as neuronal survival/differentiation factors. Accumulated evidence indicates that they play a critical role in regulation of other events of neuronal development, such as neuronal migration, synaptic formation/plasticity, and axonal growth (Lindholm et al., 1993; Thoenen, 1995; Lewin and Barde, 1996; Tucker, 2002; Lu, 2003). The biological effect of BDNF is transmitted by a receptor tyrosine kinase, TrkB, at the cytoplasmic membrane. Binding to BDNF initiates TrkB dimerization and autophosphorylation at tyrosine residues (Patapoutian and Reichardt, 2001). The phospho-tyrosine residues of TrkB recruit effector proteins and trigger signaling cascades that eventually result in gene transcription (Kaplan and Miller, 2000; Patapoutian and Reichardt, 2001). BDNF and TrkB are expressed in the cerebellum and their expression is developmentally regulated (Lindholm et al., 1997). Cerebellar granule neurons (CGNs) express both BDNF and TrkB during development (Rocamora et al., 1993; Segal et al., 1995), and BDNF/TrkB interaction promotes survival, differentiation and migration of CGNs (Segal et al., 1992; Lindholm et al., 1993; Rocamora et al., 1993; Schwartz et al., 1997; Borghesani et al., 2002). It has been proposed that ethanol neurotox-

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icity may be mediated by an aberration in the NT/receptor system (Bhave et al., 1999; Light et al., 2001, 2002; Climent et al., 2002). Disruption of transcriptional activity may be a potential mechanism of ethanol damage in a rat fetal alcohol syndrome model (Acquaah-Mensah et al., 2002). We hypothesized that ethanol may interfere with BDNF-stimulated signaling and therefore inhibits BDNF-regulated transcription activity.

The activator protein-1 (AP-1) is a transcription factor that controls the transcription of a wide array of genes involved in cell proliferation, differentiation and survival. AP-1 regulates the transcription of genes with a consensus DNA recognition sequence TGA/(C/G), designated as 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-responsive element (TRE) in their promoter region (Angel and Karin 1991). AP-1 proteins consist of a family of Jun/Fos dimers that include different Jun proteins (c-Jun, JunB, and JunD) and Fos proteins (c-Fos, FosB, Fra-1, Fra-2 and FosB2; Angel and Karin, 1991). Each of these proteins consists of a "leucine zipper," which permits Jun proteins to form homodimers or heterodimers among themselves or with Fos proteins (Angel and Karin, 1991). Interaction of AP-1 with TRE in the promoter region regulates gene transcription. In the present study, we examined the AP-1 transactivation in cultured CGNs derived from the transgenic mice expressing the AP-1 luciferase reporter gene. We sought to determine whether ethanol affects BDNF-stimulated AP-1 transactivation, and explore the mechanism(s) of ethanol action.

EXPERIMENTAL PROCEDURES

Materials

Tetracycline (Tet) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). MEK1 inhibitor (PD98059) and p38 MAPK inhibitor (SB203580) were obtained from Calbiochem (San Diego, CA, USA). PI3K inhibitor (LY294002) was purchased from Promega (Madison, WI, USA). JNK inhibitor D-JNKI1 was obtained from Alexis Biochemicals (San Diego, CA, USA).

AP-1 luciferase reporter transgenic mice and culture of CGNs

2X TRE luciferase reporter transgenic mice were originally established by Rincon and Flavell (1994). A C57BL/6 male mouse carrying the 2X TRE-luciferase transgene was crossed with DBA/ two females (SASCO, Omaha, NE, USA; Huang et al., 1997). The F1 offspring were screened by testing both the basal and TPA-induced levels of luciferase activity for the presence of the AP-1-luciferase reporter gene. Mice were housed under temperature, humidity and light controlled conditions. Food and water were available *ad libitum*. All animal protocols were approved by the ACUC of WVU and complied with the NIH guidelines for animal care and use. Amount of animals represented a minimal but adequate number for statistical analysis. Efforts were made to minimize suffering.

Cultures of CGNs were generated using a previously described method (Li et al., 2001; Luo et al., 2003). Briefly, cerebella were obtained from 5- or 6-day-old pups of AP-1-luciferase reporter transgenic mice. The expression of AP-1 reporter transgene in these pups was screened and verified by a robust up-regulation of luciferase activity in the skin following a topical application of TPA. The cerebella were minced with a sterile razor blade and suspended in 10 ml of trypsin solution (0.025%) at 37 °C. After incubation for 15 min, an equal volume of a solution containing DNAse (130 Knuitz units/ml) and trypsin inhibitor (0.75 mg/ml) was added and the tissue was sedimented by a brief (5 s) centrifugation. The tissue was dissociated by trituration, and the cell suspension was centrifuged through a 4% bovine serum albumin solution, which is a critical step for increasing neuronal viability. The cell pellet was re-suspended in Eagle's MEM containing the following supplements: 10% fetal bovine serum (FBS), 25 mM KCl, 1 mM glutamine, 33 mM glucose, and penicillin (100 units/ml)/streptomycin (100 μg/ml). Cells (seeding density of 3.2–$6.4\times10^4/cm^2$) were plated into poly-D-lysine (50 μg/ml)-coated cell culture wells or dishes. The cells were then incubated at 37 °C in a humidified environment containing 5% CO_2. Twenty-four hours after plating, cells were treated with BDNF with/without ethanol (400 mg/dl) for 14–18 h, and then harvested for assaying AP-1 activity. For determining protein kinase phosphorylation, 24 h after plating in the medium containing 10% FBS, cells were switched to a serum free medium overnight, and then treated with BDNF with/without ethanol (400 mg/dl) for 5–120 min.

Culture of human neuroblastoma cells expressing inducible TrkB

Human neuroblastoma SH-SY5Y cells expressing inducible TrkB (TB8 cells) were generated under the control of a Tet-repressible promoter element (Jaboin et al., 2002). Cells were cultured in RPMI 1640 containing 10% FBS, 2.0 mM glutamine and penicillin (100 units/ml)/streptomycin (100 μg/ml).

Ethanol exposure protocol

A method utilizing sealed containers (Luo and Miller 1997) was used to maintain ethanol levels in the culture medium. With this method, ethanol concentrations in the culture medium can be accurately maintained. A pharmacologically relevant concentration of 400 mg/dl was used in this study. In general, the concentration for *in vitro* studies is higher than those required to produce similar effects *in vivo* (Luo et al., 2001).

Cell transfection

TB8 cells were maintained in RPMI 1640 medium containing Tet (1 μg/ml; Tet-on condition). For induction of TrkB expression, cells were switched to a Tet-free medium (Tet-off condition) and cultured for 3 days. Procedure for cell transfection has been previously described (Luo et al., 2003). Briefly, cells were plated into 24-well tissue culture trays at a density of 2×10^5 cells/ml. The AP-1 luciferase reporter plasmid (−73/+63 collagenase promoter-luciferase) was previously described (Dong et al., 1995). This vector was constructed by insertion of a sequence of the collagenase promoter region (−73/+63) containing one AP-1 binding site (TGAGTCA) into the luciferase reporter vector pGL2-basic (Promega). The AP-1 luciferase reporter plasmid (0.8 μg) was incubated with a transfection solution containing 2 μl of LIPOFECTAMINE 2000 (LF2000) Reagent (Life Technologies, Rockville, MD, USA) and 98 μl of Opti-MEM reduced serum medium (Life Technologies) at room temperature for 20 min to allow the formation of DNA-LF2000 Reagent complexes. One hundred microliters of the DNA-LF2000 complex was directly added to cell culture wells containing TB8 cells and mixed with gentle rocking. The incubation occurred at 37 °C in a humidified environment containing 5% CO_2. Exposure to ethanol or BDNF was initiated 24 h after transfection. Luciferase activity was assayed at 10 h after exposure to ethanol or BDNF. For controls, cells were transfected with an empty pGL2-basic vector.

Measurement of AP-1 activity

AP-1 transactivation in CGNs and TB8 cells was determined by assaying the activity of the luciferase reporter (Ma et al., 2003a). Briefly, cells were cultured in 96-well plates (for CGNs) or 24-well plates (for TB8 cells) and grown in a medium containing 10% FBS. The plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2. For assaying AP-1 activity, sub-confluent cultures were treated with BDNF, ethanol or various protein kinase inhibitors. After treatment, cellular protein was extracted with a 1× lysis buffer supplied in the luciferase assay kit (Promega), and incubated with a luciferase substrate solution (Promega). Luciferase activity was measured with a luminometer (3010; Analytical Luminescence Laboratory, Sparks, MD, USA). AP-1 activity (luciferase activity) was calculated and expressed relative to the untreated cultures.

MTT assay

The MTT assay was employed to determine the number of viable cells in culture (Roche Molecular Biochemicals, Indianapolis, IN, USA) as previously described (Sun et al., 2002). The assay is based on the cleavage of the yellow tetrazolium salt MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide] to purple formazan crystals by metabolically active cells. Briefly, the cells were plated into 96-well microtiter plates and exposed to ethanol, BDNF or protein kinase inhibitors. Following treatment, 10 μl of MTT labeling reagent were added to each well and the plates were incubated at 37 °C for 4 h. The cultures were then solubilized and spectrophotometric absorbance of the samples was detected by a microtiter plate reader. The wavelength to measure absorbance of formazan product is 570 nm, with a reference wavelength of 750 nm.

Immunoblotting

The immunoblotting procedure for detecting phosphorylation and expression of signal proteins was performed as previously described (Luo et al., 2003). Briefly, cells were washed with PBS and lysed with RIPA buffer for 10 min. Cell lysate was transferred to microcentrifuge tubes, and centrifuged. Supernatant was collected, and protein concentration was determined. Aliquots of the protein (60 μg) were loaded onto the lanes of a sodium dodecylsulfate polyacrylamide gel (7.5–10%). The proteins were separated by electrophoresis, and transferred to nitrocellulose membranes. The membranes were washed with 5% nonfat dry milk in 0.010 M phosphate-buffered saline (pH 7.4) and 0.10% Tween-20 (TPBS) at room temperature for 1 h to block non-specific immunoreactivity. Subsequently, the membranes were incubated with primary antibodies directed against various phosphorylated protein kinases for 2 h at room temperature or overnight at 4 °C. Antibody directed against phospho-Akt was purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Antibody directed against phospho-JNKs was obtained from Promega. Antibodies directed against phospho-ERK1/2 (T202/Y204) and phospho-p38 MAPK were purchased from BD Transduction Laboratories (San Jose, CA, USA). Antibodies against TrkB and phospho-Trk were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). After two quick washes in TPBS, the membranes were incubated with a secondary antibody conjugated to horseradish peroxidase (Amersham, Arlington Heights, IL, USA) diluted at 1:2000 in TPBS for 1 h. The immune complexes were detected with the enhanced chemiluminescence method (Amersham). After detection of phosphorylated proteins, blots were stripped and re-probed with antibodies directed against non-phosphorylated forms of protein kinases (Santa Cruz Biotech). The relative amount of each protein imaged on the films was measured microdensitometrically using SigmaGel software (SPSS, Chicago, IL, USA), and normalized to non-phosphorylated forms of protein kinases.

Extraction of nuclear proteins

TB8 cells were cultured in a Tet-off condition for 2 days, and then exposed to BDNF (100 ng/ml) with or without ethanol (400 mg/dl) for 48 h. The procedure for extraction of nuclear protein was previously described (Ye et al., 1996). Briefly, cells (1×10^7) were lysed with 500 μl of lysis buffer [50 mM KCl, 0.5% Nonidet P-40, 25 mM HEPES, pH 7.8, 1.0 mM pheylmethylsulfonyl fluoride (PMSF), 10 μg/ml of leupeptin, 20 μg/ml of aprotinin and 100 μM [dl]-dithiothreitol (DTT)] on ice for 4 min. After 1 min of centrifugation at 14,000 r.p.m., the supernatants were discarded and pellets (nuclei) were collected. The nuclei were washed once with 500 μl of lysis buffer omitting Nonidet P-40, and the nuclear proteins were extracted with 300 μl of extraction buffer (500 mM KCl and 50% glycerol, 25 mM HEPES, pH 7.8, 1 mM PMSF, 10 μg/ml of leupeptin, 20 μg/ml of aprotinin and 100 μM DTT). After centrifugation at 14,000 r.p.m. for 5 min, the supernatant (nuclear protein extract) was harvested and stored at −70 °C. The protein concentration was determined by Lowry assay.

Electrophoretic mobility shift assay (EMSA)

The AP-1 binding oligonucleotide, a sequence of collagenase gene promoter (5′-ATGAGTCAGACACCTCTGGCTTTCTGGAAG-3′), was kindly provided by Dr. Jianping Ye (Louisiana State University, Baton Rouge, LA, USA). The DNA-protein binding reaction was conducted in a 24 μl reaction mixture including 1 μg of poly (dI · dC; Sigma), 3 μg of nuclear protein extract, 3 μg of BSA, 4×10^4 cpm ^{32}P-labeled oligonucleotide probe and 12 μl of reaction buffer (12% glycerol, 24 mM HEPES, pH 7.9, 8 mM Tris–HCl pH 8, 2 mM EDTA pH 8 and 1 mM DTT). In some groups, an unlabeled double-strand oligomer was added as a cold competitor. The reaction mixture was incubated for 10 min at 4 °C and the DNA-protein complexes were loaded onto a 6% non-denatured PAGE gel that had been pre-run at 200 V with a 0.5× Tris–borate–EDTA buffer for 30 min. The DNA-protein complexes were separated by electrophoresis at 200 V for 90 min, dried and placed on Kodak X-OMAT film (Eastman Kodak, Rochester, NY, USA). The film was developed after overnight exposure at −70 °C.

Statistical analysis

Differences among treatment groups were tested using analysis of variance. Differences in which the *P* value was less than 0.05 were considered statistically significant. In cases where significant differences were detected, specific post hoc comparisons between treatment groups were examined with Student-Newman-Keuls tests.

RESULTS

Ethanol inhibits BDNF-stimulated AP-1 activity in cultured neuronal cells

The effect of BDNF and TPA on AP-1 activity was examined in CGNs cultured in medium containing 10% FBS; serum is important for the survival of cultured CGNs. Inclusion of serum in the culture medium was to minimize the influence of cell viability on AP-1 activity. The expression of AP-1 reporter transgene in CGNs was confirmed by their response to TPA treatment. An initial experiment indicated that the maximal activation of AP-1 occurred between 12 and 24 h following BDNF treatment, and persisted for 48 h (data not shown). In this experiment, CGNs were exposed to BDNF or TPA for 14–18 h. As shown in Fig. 1, TPA stimulated AP-1 transactivation in cultured CGNs. BDNF

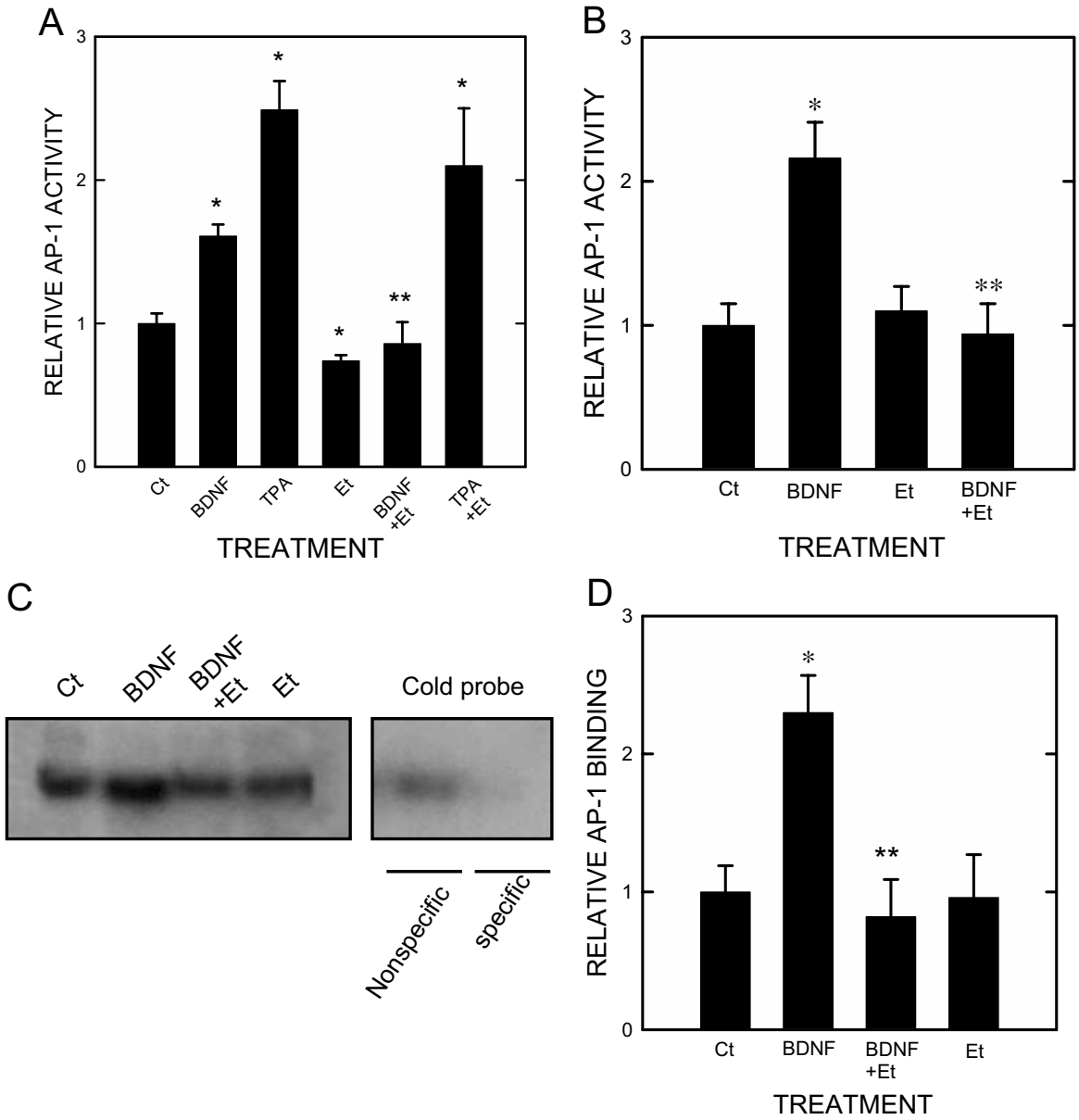

Fig. 1. Effect of ethanol on BDNF-mediated AP-1 transactivation in cultured CGNs and human neuroblastoma over-expressing TrkB. (A) CGNs were isolated from transgenic mice expressing AP-1-luciferase reporter construct and cultured as described under the Experimental Procedures. Cells were grown in a medium containing 10% FBS, and treated with BDNF (100 ng/ml, 14–18 h) or TPA (60 ng/ml, 14–18 h) with or without ethanol (Et; 400 mg/dl). AP-1 transactivation (luciferase activity) was measured and expressed relative to the untreated cultures as described under the Experimental Procedures. Each data point (±S.E.M.; bars) is the mean of four independent experiments. * $P<0.05$, significant difference from untreated cultures (Ct); ** $P<0.05$, significant difference from paired, BDNF-treated group. (B) The expression of TrkB in TB8 cells was induced by the removal of Tet in the medium for 3 days. Cells were then transfected with AP-1-luciferase reporter plasmid. Twenty-four hours after transfection, cells were treated with BDNF (0 or 100 ng/ml) with or without Et (400 mg/dl). Ten hours after treatment, AP-1 transactivation (luciferase activity) was measured and expressed relative to the untreated cultures. Each data point (±S.E.M.; bars) is the mean of four independent experiments. * $P<0.05$, significant difference from the Ct; ** $P<0.05$, significant difference from paired, BDNF-treated group. (C) TB8 cells over-expressing TrkB were treated with BDNF (100 ng/ml) with or without Et (400 mg/dl) for 48 h. Nuclear protein was extracted and AP-1 binding activity was determined by an EMSA as described under the Experimental Procedures. Equal loading of nuclear proteins was determined by Coomassie Blue-staining after detection of AP-1 binding activity. (D) Quantification of AP-1 binding activity. The relative amount of AP-1 binding activity (results in the panel B) was quantified microdensitometrically. Each data point (±S.E.M.; bars) is the mean of three independent experiments. * $P<0.05$, significant difference from the Ct. ** $P<0.05$, significant difference from paired, BDNF-treated group.

also significantly increased AP-1 activity, but to a lesser extent. To rule out the possibility that change in AP-1 activity resulted from an alteration in cell survival, MTT assay was employed to determine cell viability. Treatment of TPA (60 ng/ml), BDNF (100 ng/ml) or ethanol (400 mg/dl) for 18 h did not significantly alter cell viability (data

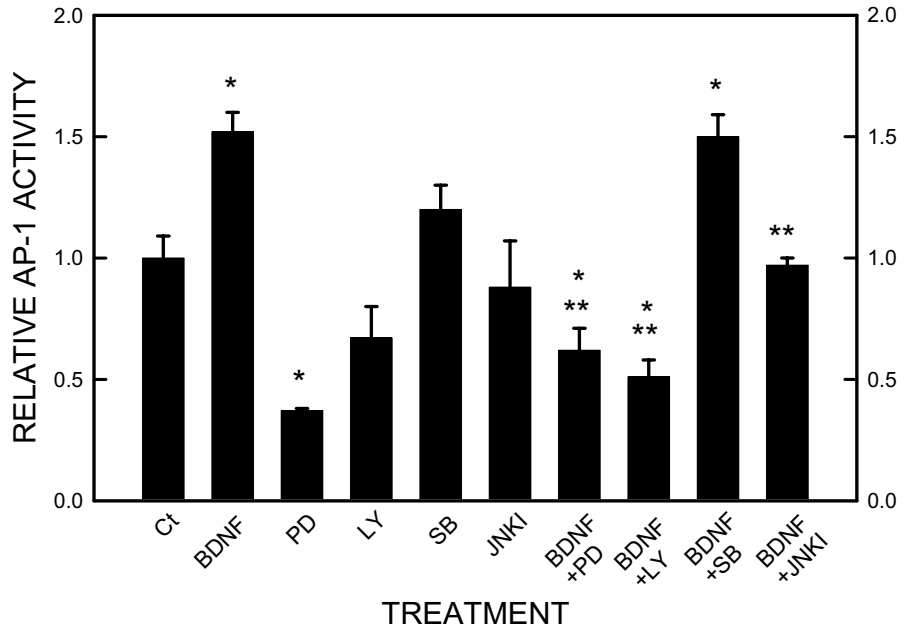

Fig. 2. Effect of specific protein kinase inhibitors on BDNF-mediated AP-1 transactivation. CGNs were isolated from transgenic mice expressing AP-1-luciferase reporter construct and cultured in a medium containing 10% FBS. Cells were treated with BDNF (100 ng/ml) with or without various protein kinase inhibitors for 14–18 h. PD98059 (PD; 50 μM) is a MEK1 inhibitor; LY294002 (LY; 10 μM) is a PI-3K inhibitor; SB203580 (SB; 10 μM) is a p38 MAPK inhibitor; D-JNKI1 (JNKI; 1 μM) is a JNK inhibitor. AP-1 transactivation (luciferase activity) was measured and expressed relative to the untreated cultures as described in the Experimental Procedures. Each data point (±S.E.M.; bars) is the mean of four independent experiments. * $P<0.05$, significant difference from the untreated cultures (Ct); ** $P<0.05$, significant difference from paired, BDNF-treated group.

not shown). Ethanol exposure induced a modest and statistically significant ($P<0.05$) inhibition of basal AP-1 activity (activity not stimulated by BDNF or TPA), and blocked BDNF-stimulated AP-1 activity (Fig. 1A). However, ethanol did not significantly affect TPA-induced AP-1 activation. Since CGNs express TrkB, but not TrkA (Courtney et al., 1997), ethanol-mediated inhibition must result from the disruption of TrkB signaling.

To verify that TrkB signaling and AP-1 activity were the target of ethanol action, we examined the effect of ethanol on a human neuronal cell line, SH-SY5Y cells expressing inducible TrkB. SH-SY5Y cells express very low levels of TrkB, and barely respond to BDNF. With a Tet-regulated system (TB8 cells), high expression of TrkB can be induced in SH-SY5Y by removal of Tet in culture medium (Tet-off; Jaboin et al., 2002). By transient transfection of a AP-1 luciferase reporter, we demonstrated that BDNF significantly activated AP-1 and ethanol exposure eliminated BDNF-mediated AP-1 transactivation in Tet-off TB8 cells expressing grown in a serum free medium (Fig. 1B). Similar blocking effect of ethanol was observed in TB8 cells grown in a medium contained 5% serum (data not shown). This finding was consistent with the results obtained from CGNs. As a negative control, BDNF and ethanol had no effect on Tet-on TB8 cells (data not shown).

With the expression of AP-1 luciferase reporter transgene, we were able to demonstrate that BDNF induced AP-1 transactivation and that ethanol blocked BDNF-mediated activity in CGNs and SH-SY5Y cells expressing TrkB. To verify that BDNF and ethanol affected the binding of the AP-1 protein complex to DNA, we employed EMSA to examine the binding activity of the AP-1 protein complex to an oligonucleotide containing AP-1 binding sites. As shown in Fig. 1C and D, BDNF significantly increased AP-1 binding activity in TB8 cells expressing TrkB. The binding activity was eliminated by incubation of an excess amount of cold AP-1 oligonucleotide (unlabeled oligonucleotide), but not altered by a cold, unrelated oligonucleotide, indicating that the binding was specific for the AP-1 transcription factor. Ethanol blocked BDNF-stimulated AP-1 DNA binding activity. This result agreed with the observation showing that ethanol eliminated BDNF-mediated AP-1 transactivation (Fig. 1A and B).

PI3K and JNKs are involved in BDNF-stimulated AP-1 activity

The activity of AP-1 is regulated by multiple signaling pathways. Among these pathways, phosphatidylinositol 3-kinase (PI3K)- and MAPK-mediated signal transduction play a critical role. To delineate the signal pathways that regulate BDNF stimulation of AP-1 activity, we employed various specific inhibitors to block PI3K- and MAPK-mediated signaling. We have previously demonstrated that these inhibitors specifically block respective kinases at the concentrations applied (Ma et al., 2003b). CGNs were isolated from mice that were tested positive for expression of AP-1-luciferase reporter transgene. To minimize the influence of cell viability on AP-1 activity, CGNs were maintained in a medium containing 10% FBS. Cells were treated with BDNF with/without specific protein kinase inhibitors, and AP-1 activity was determined. As shown in

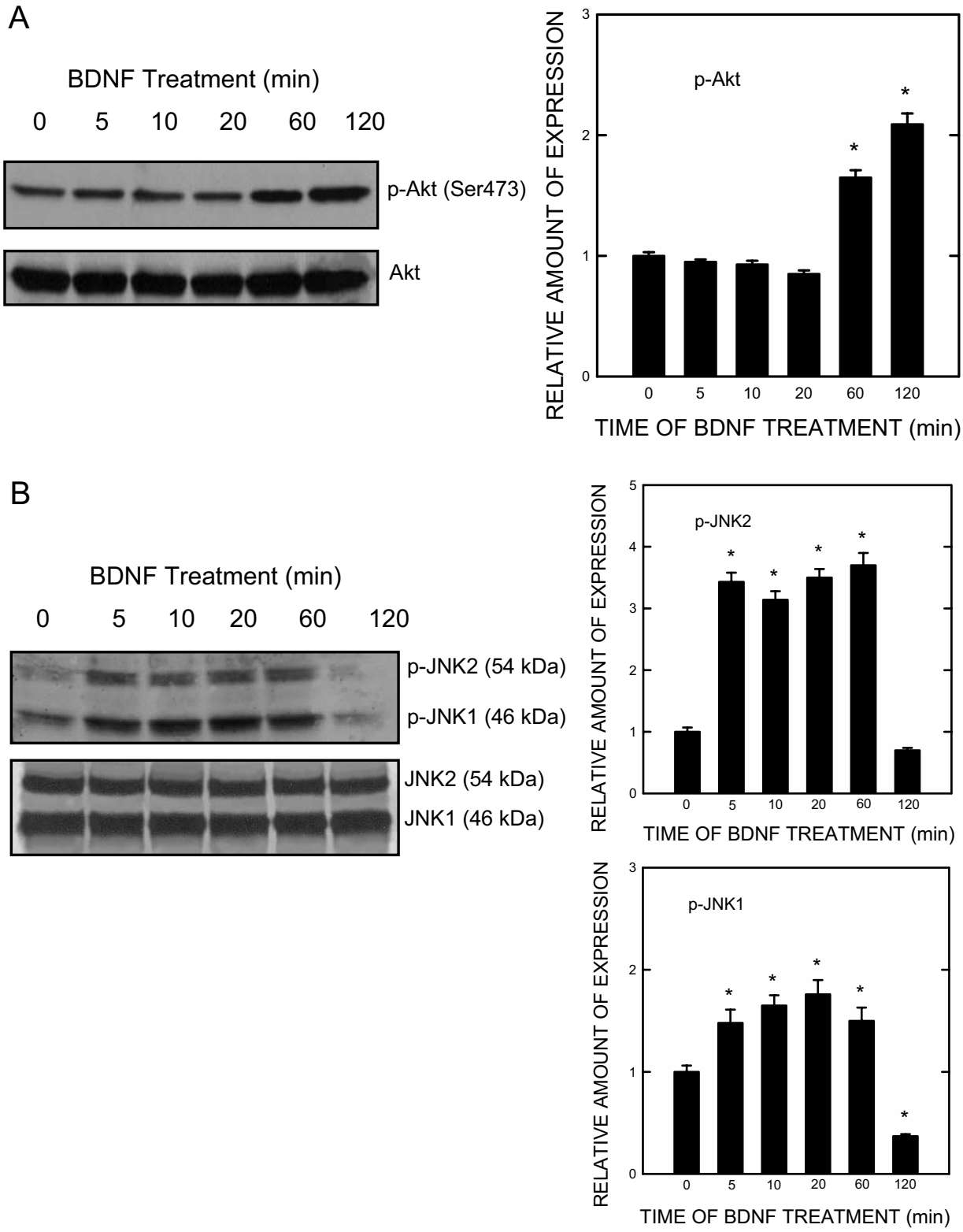

Fig. 3. BDNF-induced phosphorylation of Akt and JNKs in CGNs. CGNs were isolated from transgenic mice expressing AP-1-luciferase reporter construct and cultured in a medium containing 10% FBS. After attachment, cells were cultured in a serum free medium for 14–18 h. Cells were treated with BDNF (100 ng/ml) for various durations (5–120 min), and phosphorylation of Akt (A) and JNKs (B) was examined by immunoblots. Left panel: A representative immunoblot showing BDNF induced phosphorylation. Right panel: The relative amount of phosphorylated proteins was quantified microdensitometrically and normalized to non-phosphorylated forms of the kinases. Each data point (±S.E.M.; bars) is the mean of four independent experiments. * $P<0.05$, significant difference from the untreated cultures (time 0).

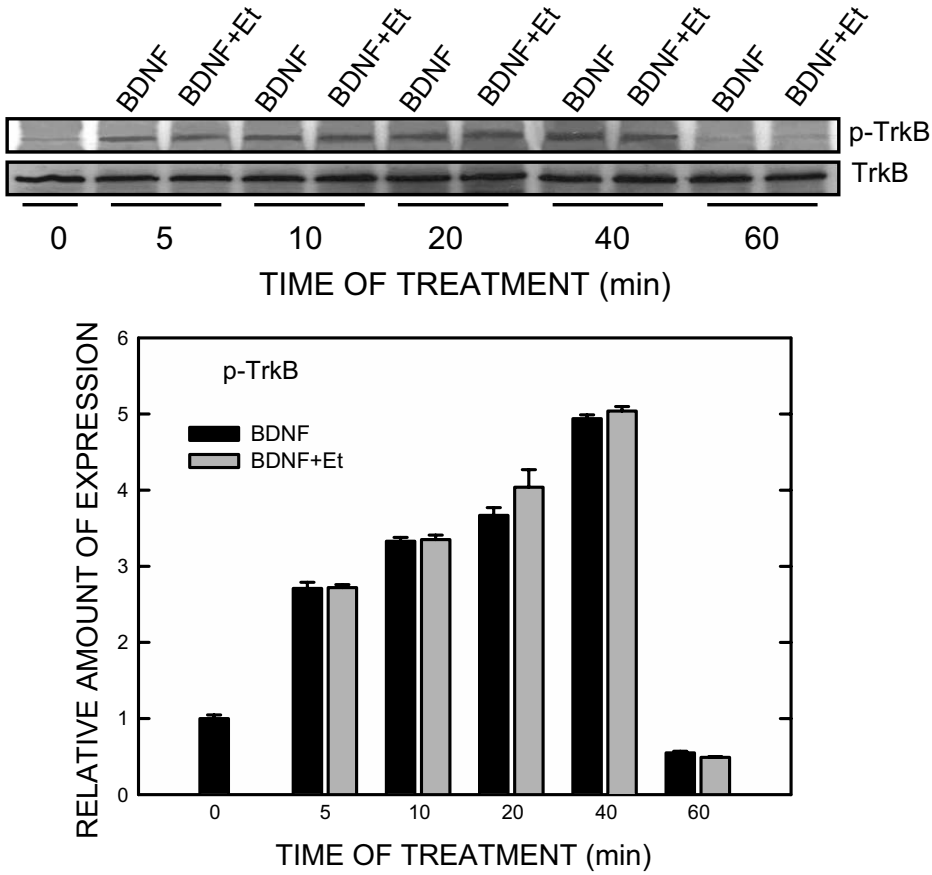

Fig. 4. Effect of ethanol on autophosphorylation of TrkB. TB8 cells over-expressing TrkB was cultured in a serum free medium overnight, and then treated with BDNF (100 ng/ml) with or without ethanol (Et; 400 mg/dl). The phosphorylation of TrkB was examined by immunoblots (top panel), and relative amount of autophosphorylation was quantified microdensitometrically and normalized to non-phosphorylated form of TrkB (bottom panel). The experiment was replicated three times.

Fig. 2, SB203580 (p38 MAPK inhibitor), LY294002 (PI-3K inhibitor) and D-JNKI1 (JNK inhibitor) had little effect on the basal levels of AP-1 activity, while PD98059 (MEK1 inhibitor) significantly inhibited basal level of AP-1 activity. This inhibition probably resulted from the blockage of serum-stimulated AP-1 activity. Although LY294002 and D-JNKI1 did not significantly alter the basal levels of AP-1 activity, they blocked BDNF-stimulated AP-1 transactivation. The effect of these inhibitors on AP-1 activity did not result from a change in cell viability since exposure to these inhibitors (14–18 h) did not significantly alter cell viability as assayed by MTT (data not shown).

The experiments using specific inhibitors for protein kinases suggested that PI3K and JNKs were critical mediators of BDNF-stimulated AP-1 transactivation. We next sought to verify whether BDNF indeed activated PI3K and JNKs. As shown in Fig. 3, BDNF significantly increased the phosphorylation of Akt, a substrate of PI3K, as well as JNKs (JNK1 and JNK2) without affecting their levels of non-phosphorylated forms of proteins in cultured CGNs. It was interesting to note that the phosphorylation of JNK1 was modestly inhibited by exposure to BDNF for 120 min. However, BDNF had little effect on the phosphorylation of ERKs and p38 MAPK (data not shown).

Ethanol does not affect the expression and autophosphorylation of TrkB

The interference with BDNF signaling could result from the effect of ethanol on the expression and/or activation of TrkB. We sought to investigate the effect of ethanol on TrkB expression and autophosphorylation. The CGNs were cultured in a serum free medium overnight and exposed to BDNF (100 ng/ml) with/without ethanol (400 mg/dl) for 5–120 min. The phosphorylation on TrkB was determined by immunoblot using anti-phospho-Trk antibody (Santa Cruz Biotech), and the expression of TrkB was examined by immunoblot using a monoclonal anti-TrkB antibody (Santa Cruz Biotech). The expression levels of TrkB and BDNF-induced autophosphorylation were low in cultured CGNs, and we showed that ethanol had little effect on TrkB expression and BDNF-induced autophosphorylation (data not shown). Due to low expression levels of TrkB and weak autophosphorylation of TrkB, it was difficult to unequivocally evaluate the activation of TrkB in CGNs. Therefore, TB8 cells over-expressing TrkB were used because BDNF-induced TrkB phosphorylation was much more evident in these cells. As shown in Fig. 4, BDNF induced autophosphorylation of TrkB at 5 min, and

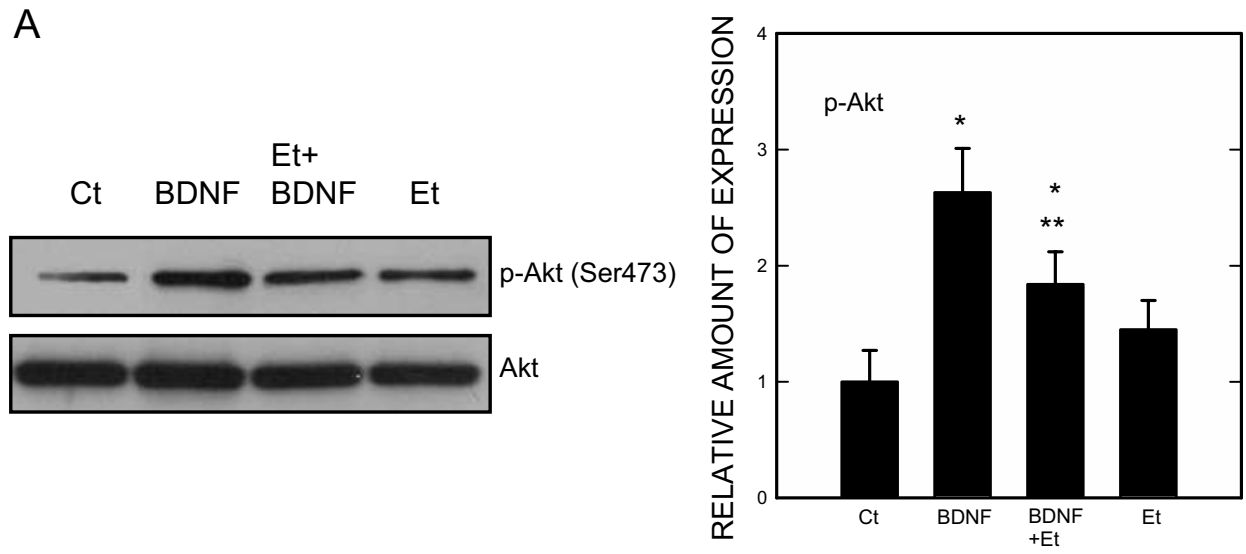

Fig. 5. Effect of ethanol on BDNF-mediated phosphorylation of Akt and JNKs in CGNs and TB8 cells. CGNs were isolated from transgenic mice expressing AP-1-luciferase reporter construct and cultured in a medium containing 10% FBS. Twenty-four hours after plating, medium was replaced with fresh medium containing no serum. Cells were maintained in the serum free medium overnight, and treated with BDNF (100 ng/ml) with or without ethanol (Et; 400 mg/dl) for 60 min. The phosphorylation of Akt (A) and JNKs (B) in CGNs was examined by immunoblots. Left panel: A representative immunoblot showing BDNF-induced phosphorylation. Right panel: The relative amount of phosphorylated proteins was quantified microdensitometrically and normalized to non-phosphorylated forms of the kinases. Each data point (±S.E.M.; bars) is the mean of four independent experiments. * $P<0.05$, significant difference from untreated cultures (Ct); ** $P<0.05$, significant difference from paired, BDNF-treated group. TB8 cells over-expressing TrkB were cultured in a serum free medium overnight, and then treated with BDNF (100 ng/ml) with or without Et (400 mg/dl). The phosphorylation of Akt (C) and JNKs (D) in TB8 cells was examined by immunoblots (top panel), and relative amount of expression was quantified microdensitometrically and normalized to non-phosphorylated forms of the kinases (bottom panel). The experiment was replicated three times.

sustained for at least 40 min. Ethanol exposure did not alter either the phosphorylation or expression of TrkB at each time point examined. These results suggested the target of ethanol action was downstream of TrkB.

Ethanol inhibits BDNF-mediated activation of PI3K and JNKs

Since PI3K/Akt and JNKs mediated BDNF-stimulated AP-1 transactivation, we sought to determine whether ethanol blocked these signaling pathways. As shown in Fig. 5A and B, ethanol significantly inhibited BDNF-induced phosphorylation of Akt and JNKs in cultured CGNs without affecting their expression levels. It was noted that ethanol significantly increased basal phosphorylation of JNKs (phosphorylation not stimulated by BDNF). This may represent a cellular stress response to ethanol exposure. Similarly, ethanol eliminated BDNF-regulated Akt phosphorylation in TB8 cells over-expressing TrkB (Fig. 5C). Ethanol, however, did not significantly affect BDNF-mediated phosphorylation of JNKs in TB8 cells expressing TrkB (Fig. 5D). This finding was different from that obtained from CGNs. The discrepancy may be due to the difference in the genetic background and signaling components that these cells inherited.

DISCUSSION

The present study provides an important insight into the intracellular signaling pathways that regulate BDNF-stimulated gene transcription and the potential site of ethanol action. Using a transgenic mouse model expressing AP-1 luciferase reporter, we demonstrate that BDNF stimulates AP-1 transactivation in CGNs. This observation is validated by the study using a human neuroblastoma cell line over-expressing TrkB, a high-affinity receptor for BDNF. BDNF induces AP-1 transactivation as well as increases the binding activity of AP-1 proteins to the specific DNA sequence in these cells. BDNF-stimulated AP-1 activation is mediated by PI3K/Akt and JNK pathways; blocking these pathways inhibits BDNF-enhanced AP-1 transactivation. Ethanol does not alter either the expression or autophosphorylation of TrkB; however, it blocks BDNF-mediated activation of PI3K/Akt and JNKs, as well as AP-1 activation.

Regulation of CGN development by BDNF

BDNF and its receptor TrkB are expressed in the developing cerebellum, and the levels of expression are developmentally regulated (Lindholm et al., 1993). CGNs, the most abundant neurons in the CNS, are susceptible to ethanol exposure *in vitro* and *in vivo*. BDNF and its receptors are expressed in the CGNs, and regulate the development of CGNs (Lindholm et al., 1993). For example, BDNF is shown to mediate survival (Nonomura et al., 1996; Dudek et al., 1997; Skaper et al., 1998; Foulstone et al., 1999), differentiation (Gao et al., 1995; Lin et al., 1998), neurite extension (Gao et al., 1995; Segal et al., 1995), and migration (Gao et al., 1995; Tanaka et al., 2000; Borghesani et al., 2002) of CGNs.

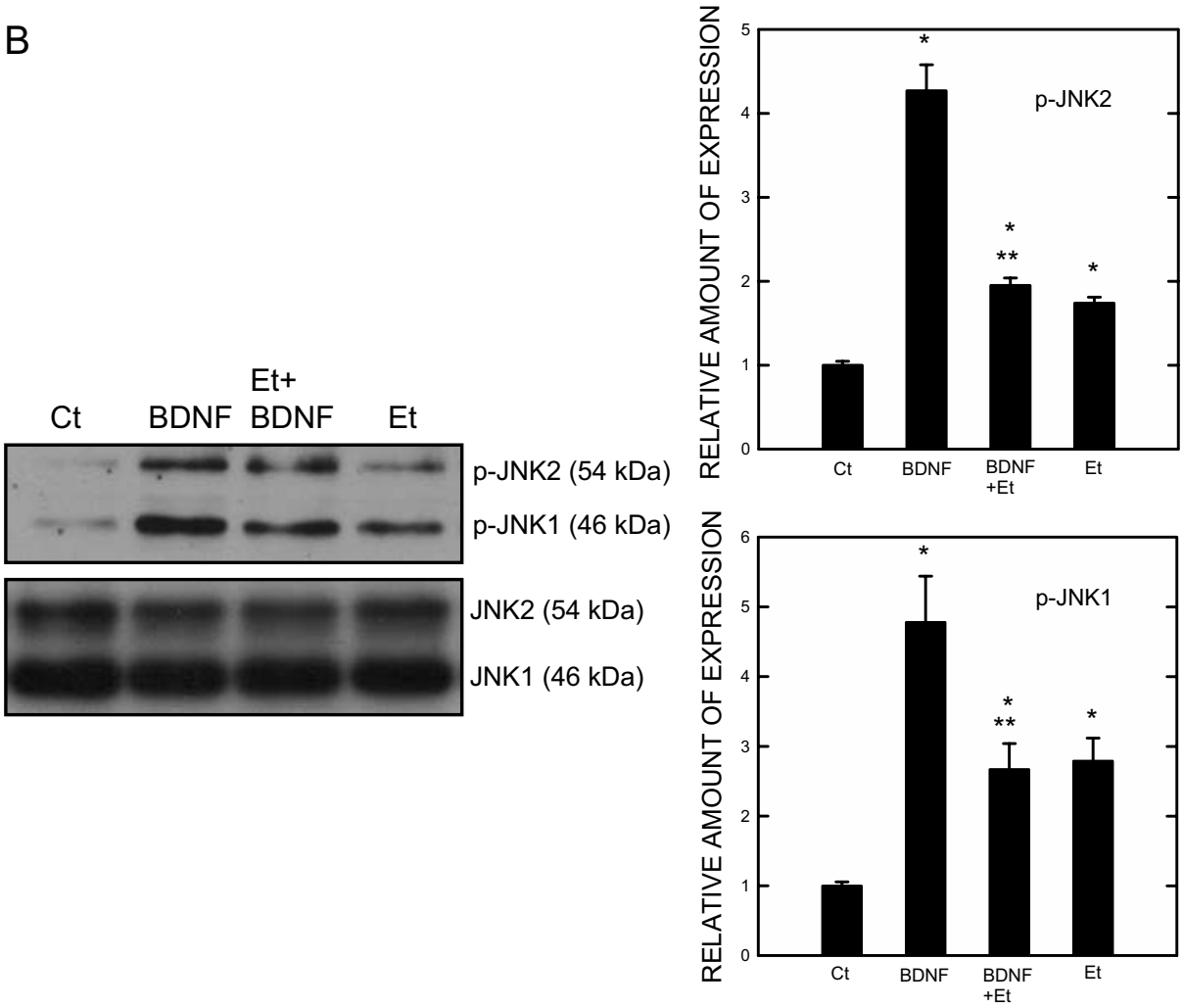

Fig. 5. (B).

Many effects of BDNF are mediated by the control of gene expression. For example, the survival promoting effect of BDNF may be mediated by up-regulation of anti-apoptotic genes or suppression of pro-apoptotic genes (Bonni et al., 1999; Schabitz et al., 2000) although post-transcriptional or posttranslational mechanisms are also observed (Datta et al., 1997; Suzuki and Koike, 1997; Bonni et al., 1999). BDNF-regulated gene expression is also directly associated with neuronal differentiation (Carnahan and Nawa, 1995; Lin et al., 1998).

AP-1 is a transcription factor that controls many important cellular processes, such as cell proliferation, differentiation and survival; the regulation of these processes is mediated by gene expression (Shaulian and Karin, 2002). For example, AP-1 is involved in the control of the cell cycle by regulating the expression of cyclin A and cyclin D1, which contain AP-1 binding sites in their promoter region (Herber et al., 1994; Albanese et al., 1995; Sylvester et al., 1998). AP-1 can also affect cell survival by regulating the expression of anti-apoptotic genes, such as bcl-3, bcl-x (Rebollo et al., 2000; Sevilla et al., 2001), as well as pro-apoptotic genes, such as p53 and FasL (Kasibhatla et al., 1998; Schreiber et al., 1999; Rebollo et al., 2000). AP-1 may either positively or negatively regulate cell survival depending on the cell type or developmental status (Hilberg et al., 1993; Schreiber et al., 1999; Behrens et al., 2000).

We demonstrate that BDNF can stimulate AP-1 transactivation in the CGNs derived from transgenic mice expressing an AP-1 luciferase reporter construct. This observation is consistent with a previous report showing that BDNF stimulates AP-1- and cyclic AMP-responsive element-dependent transcriptional activity in cultured CGNs (Gaiddon et al., 1996; Kuan et al., 1999). Therefore, it can be concluded that one mechanism by which BDNF regulates gene transcription is through activating AP-1. This transgenic mouse model has been previously used to evaluate AP-1 transactivation in T lymphocytes, skin, lung and bronchial tissues (Rincon and Flavell, 1994; Huang et al., 1997; Kuan et al., 1999; Ding et al., 1999). The transgene of the AP-1-luciferase reporter driven by a rat pro-

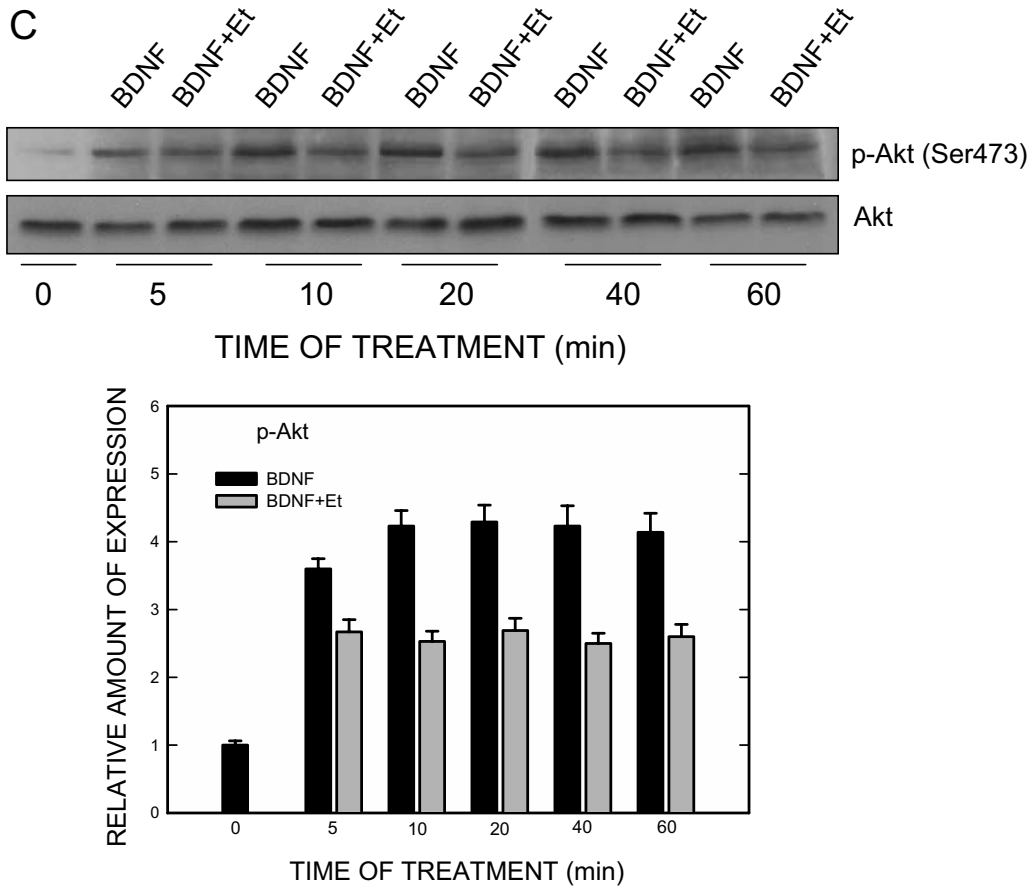

Fig. 5. (C).

lactin promoter appears ubiquitously expressed. Prolactin is expressed in the CNS including the cerebellum (Emanuele et al., 1992; Kuan et al., 1999). This study shows that BDNF and TPA can induce AP-1 transactivation in CGNs, indicating that this transgenic mouse model is also valuable in assessing AP-1 activation in CNS neurons.

Intracellular signaling for BDNF-mediated AP-1 activation

Interaction between BDNF and TrkB initiates a complex signaling cascade, including the PI3K/Akt and MAPK pathways. It has been demonstrated that PI3K/Akt plays an important role in BDNF-mediated neuroprotection and survival in CGNs (Duke and Cohen, 1986; Nonomura et al., 1996; Skaper et al., 1998; Bhave et al., 1999; Foulstone et al., 1999; Kuan et al., 1999; Daily et al., 2001). We show here that BDNF enhances phosphorylation of Akt in both CGNs and TB8 cells, indicating the activation of PI3K/Akt pathway. However, the time sequence for BDNF-induced Akt phosphorylation is different in the CGNs and TB8 cells. In TB8 cells, maximal Akt phosphorylation occurs at 10 min after BDNF treatment, while in the CGNs, the activation is not evident until 60 min following BDNF exposure. The mechanisms for delayed Akt activation in the CGNs are unknown. It suggests that Akt is indirectly regulated by BDNF in the CGNs. Among MAPKs, ERKs are mainly activated by growth factors and involved in the regulation of cell proliferation, while JNKs and p38 MAPK are most potently activated by environmental stresses (Marshall, 1995). BDNF-induced activation of JNKs is somewhat surprising because JNKs are usually activated by cellular stress and are associated with cell death (Lei and Davis, 2003; Putcha et al., 2003). However, activation of JNKs may also promote neuronal survival, and it appears that the pro-apoptotic and anti-apoptotic effect is dependent on the cell type and stage of development (Ham et al., 1995; Kuan et al., 1999; Jezierski et al., 2001). In fact, a recent study indicates that JNKs mediate neuroprotection in CGNs (Daily et al., 2001). Although BDNF may activate ERKs in other model systems and mediate neuronal survival and neuroprotection (Hetman et al., 1999; Klocker et al., 2000 Climent et al., 2002), we do not observe BDNF-induced phosphorylation of ERK and p38 MAPK in cultured mouse CGNs.

Our study shows that PI3K/Akt and JNKs are important signaling components responsible for BDNF-mediated AP-1 transactivation in CGNs; blocking either pathway eliminates BDNF-stimulated AP-1 transactivation. Furthermore, PI3K/Akt may mediate the basal AP-1 activity which is stimulated by serum. These results are supported by a

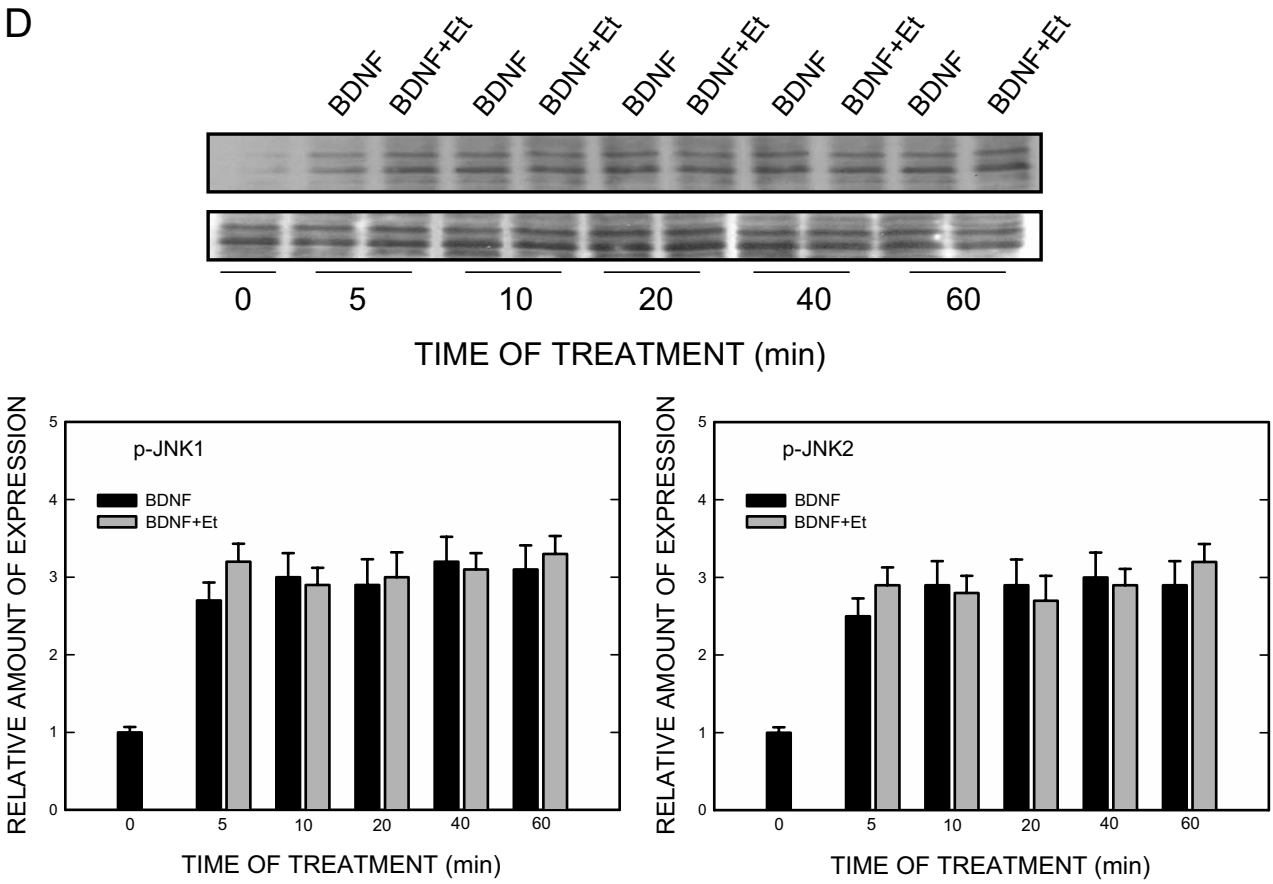

Fig. 5. (D).

recent report showing that dual activation of PI3K/Akt and JNK pathways is required for neuroprotection in CGNs, and blockage of either pathway reduces AP-1 binding activity (Daily et al., 2001). Inhibition of ERK activation by PD98059 reduces the basal AP-1 activity and blocks BDNF-stimulated AP-1. Based on our finding that BDNF does not activate ERKs in CGNs, it can be deduced that ERKs are primarily responsible for AP-1 activity regulated by serum. It appears that p38 MAPK is not involved in either the basal or BDNF-stimulated AP-1 activity.

Potential site of ethanol action

It has been demonstrated that ethanol may affect the NT system (Baek et al., 1996; Luo et al., 1996, 1997; Dohrman et al., 1997; Luo and Miller 1998; Seabold et al., 1998; Bhave et al., 1999; Heaton et al., 2000; Light et al., 2001, 2002; Climent et al., 2002). Most of these studies evaluate the expression levels of NTs/receptors after chronic ethanol exposure *in vitro* and *in vivo*, and suggest that the weakened neurotrophic support may result from an alteration in the expression of NTs/receptors. Our study shows that ethanol can interfere with BDNF signaling and block BDNF-stimulated AP-1 activity without affecting the expression/autophosphorylation of its receptor, indicating that ethanol targets downstream components of TrkB. It has been shown that ethanol can also block insulin-stimulated PI3K/Akt activation in cerebellar neurons (de la Monte and Wands, 2002), suggesting the effect of ethanol may not be specific to BDNF. In general, CGNs and TB8 cells respond similarly to BDNF and ethanol; BDNF activates PI3K/Akt, JNKs and AP-1, and ethanol inhibits BDNF-stimulated PI3K/Akt phosphorylation as well as AP-1 activation in these cells. However, some differences are observed between the two cells. For example, ethanol inhibits basal AP-1 activity and BDNF-mediated JNK phosphorylation in CGNs but not TB8 cells. CGNs were cultured under a depolarization condition (25 mM KCl) which is important for CGN survival. It is shown that chronic depolarization increases BDNF expression in CGNs (Condorelli et al., 1998). It is likely that inhibition of endogenous BDNF by ethanol exposure results in a decrease in basal AP-1 activity. The differential effect of ethanol on JNK activation between CGNs and TB8 cells may be due to the difference in the genetic background and signaling components that these cells inherited. It has been demonstrated that sensitivity to ethanol varies among cells (Luo and Miller, 1997; Ma et al., 2003b).

The mechanisms underlying ethanol blockage of BDNF-mediated activation of PI3K/Akt and JNKs are currently unknown. Activated TrkB associates with distinct

adaptor proteins and downstream effectors, such as, Shc, Grb2, PLCγ and PI3K (Atwal et al., 2000; Minichiello et al., 2002). It is likely that ethanol impairs the recruitment of these downstream adaptor/effector proteins and disrupts interaction between TrkB and these adaptors/effectors. This possibility is currently under investigation. Inhibition of BDNF-stimulated AP-1 activation implies that ethanol may impair BDNF-mediated gene transcription. Our recent study shows that ethanol selectively down-regulates the expression of certain cyclins, such as cyclin A and D, in the developing cerebellum and cultured CGNs (Li et al., 2001, 2002). These cyclins contain AP-1 binding sites in their promoter region. Thus, our study provides an insight into the molecular mechanisms of ethanol damage to the developing CNS.

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