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Lithium induces brain-derived neurotrophic factor and activates TrkB in rodent cortical neurons: An essential step for neuroprotection against glutamate excitotoxicity

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Abstract

Mechanisms underlying the therapeutic effects of lithium for bipolar mood disorder remain poorly understood. Recent studies demonstrate that lithium has neuroprotective actions against a variety of insults in vitro and in vivo. This study was undertaken to investigate the role of the brain-derived neurotrophic factor (BDNF)/TrkB signaling pathway in mediating neuroprotection of lithium against glutamate excitotoxicity in cortical neurons. Pretreatment with either lithium or BDNF protected rat cerebral cortical neurons from glutamate excitotoxicity. The duration of treatment required to elicit maximal neuroprotection by BDNF (1 day) was much shorter than that by lithium (6 days). K252a, an inhibitor of Trk tyrosine kinases, and a BDNF neutralizing antibody suppressed the neuroprotective effect of lithium. Treatment of cortical neurons with lithium increased the cellular BDNF content in 3 days and the phosphorylation of TrkB at Tyr490 in 5 days, suggesting that long-term lithium administration enhances BDNF expression/secretion, leading to the activation of TrkB receptor. Lithium failed to protect against glutamate excitotoxicity in cortical neurons derived from homozygous and heterozygous BDNF knockout mice, although lithium fully protected cortical neurons prepared from wild type mice littermates. Taken together, these data suggest that the BDNF/TrkB pathway plays an essential role in mediating the neuroprotective effect of lithium.
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Keywords: Lithium; BDNF; TrkB; Excitotoxicity; Neuroprotection; Cerebral cortical neuron

1. Introduction

Lithium was discovered more than half a century ago to be effective for the treatment of bipolar mood disorder and remains one of the primary drugs used for treating this disease. Despite intensive research, neither the etiology of bipolar disorder nor the therapeutic mechanism of lithium is well understood. One of the recently reported actions of lithium is to protect against apoptosis induced by a variety of insults in cultured neurons and neurally related cell lines (Chuang et al., 2002). These apoptotic insults include growth factor withdrawal (Bhat et al., 2000), β-amyloid administration (Alvarez et al., 1999; Wei et al., 2000), heat shock exposure (Bijur et al., 2000), and glutamate treatment (Nonaka et al., 1998). In experimental animal models, lithium reduces irradiation-induced cerebellar degeneration (Inouye et al., 1995), natural death of chick ciliary ganglion neurons (Ikonomov et al., 2000), focal ischemia-induced infarction in rat brains (Nonaka and Chuang, 1998), and quinolinic-acid induced lesions in rat striatum (Wei et al., 2001).

In an attempt to elucidate molecular and cellular

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mechanisms underlying the neuroprotective effects of lithium, we have focused our studies on glutamate-induced excitotoxicity, which has been strongly implicated in the pathogenesis of a variety of neurogenerative diseases. Using primary cultures of rat cerebellar granule cells and cerebral cortical neurons, we have reported that lithium pretreatment robustly protects against glutamate-induced apoptosis, which is almost exclusively mediated by N-methyl-D-asparate (NMDA) receptors (Hashimoto et al., 2002; Nonaka et al., 1998). These neuroprotective effects are long-lasting, occur at therapeutic concentrations (approximately 0.4–1.2 mM in the plasma) of this drug and require at least 2–3 days of pretreatment with maximal effects observed after 6–7 days. The mechanisms underlying lithium neuroprotection involve inhibition of NMDA receptors (Hashimoto et al., 2002; Nonaka et al., 1998), activation of survival-promoting factors such as the phosphatidylinositol 3-kinase (PI 3-kinase)/Akt signaling pathway (Chalecka-Franaszek and Chuang, 1999), suppressed expression of proapoptotic proteins, p53 and Bax (Chen and Chuang, 1999) and enhanced expression of the cytoprotective protein, Bcl-2 (Chen et al., 1999; Chen and Chuang, 1999). Despite this information, the upstream mechanisms and molecular links among these multiple neuroprotective mechanisms remain to be identified.

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, plays a prominent role in regulating neuronal development, survival and plasticity (Huang and Reichardt, 2001; McAllister et al., 1999; Sofroniew et al., 2001). BDNF through binding to its receptor, TrkB, causes receptor dimerization, kinase activation and phosphorylation of multiple tyrosine residues in the TrkB receptor. Phosphorylation of the tyrosine at position 490 results in the activation of the PI 3-kinase/Akt signaling pathway through the recruitment of the adaptor protein, shc (Patapoutian and Reichardt, 2001). Shc also activates Ras which mediates the activation of both the PI 3-kinase pathway and the mitogen-activated protein kinase (MAPK)/ERK pathway. PI 3-kinase/Akt activation has been shown to delay the onset of p53-mediated apoptosis (Sabbatini and McCormick, 1999) and to enhance the expression of Bcl-2 (Skorski et al., 1997). In addition, the activated TrkB receptor recruits PLC-γ1, causing phophorylation of activation of this phospholipase species (Patapoutian and Reichardt, 2001). Since BDNF/TrkB mediates many of the molecular events induced by lithium, we investigated whether the expression of BDNF and the activation of TrkB are necessary for lithium-induced protection against glutamate-induced excitotoxicity in cortical cultures prepared from embryonic rodents.

2. Methods

2.1. Animals and chemicals

All procedures employing experimental rats were performed in compliance with National Institutes of Health Guidelines for the care and use of laboratory animals. All chemicals were obtained from Sigma Chemical Co (St. Louis, MO), except that BDNF neutralizing antibody was from R & D Systems (Minneapolis, MN).

2.2. Primary cultures of rat and mice cerebral cortical neurons and drug treatments

Primary cultures of rat cerebral cortical neurons were prepared from 17-day-old embryonic rats and 18-day-old embryonic BDNF mutant mice and cultured as described previously (Hashimoto et al., 2000; Kashiwagi et al., 1998). A BDNF knockout mouse colony was raised from two pairs of BDNF+/-mice with a C57BL/6 background (Ernfors et al., 1994). These heterozygous mice, referred to as BL/6/BDNF+/-, were then crossed to obtain littermates of wild type (BDNF+/+), BDNF+/-, and BDNF−/−genotypes for the preparation of cortical neurons.

Cells were plated at a density of 4.2×10^5 cells/cm^2 on polyethyleneimine-precoated 96-well plates or 6-well plates depending on the purpose of the experiments. Cultures were maintained in serum-free B27/Neurobasal medium (Life Technologies, Rockville, MD) in a humidified atmosphere (5% CO_2, 95% air) at 37 °C. More than 95% of the cells present on day 5 in vitro were differentiated into neurons, as characterized by the appearance of long neurites expressing neurofilament protein (Kashiwagi et al., 1998). Routinely, cortical neurons on day 9 in vitro were treated with 1 mM lithium chloride or its vehicle and maintained for six days in cultures. Cortical neurons on day 14 in vitro were treated with 50 ng/ml of BDNF or its vehicle and maintained for 1 day in cultures. Glutamate (8 μM) was then added to the culture medium on day 15 in vitro and cell viability was determined 24 h later. The drug addition, cell viability measurement and other determinations were performed by different individuals and were done blindly.

2.3. Measurement of neurotoxicity

Cortical neuronal cultures were plated on 96-well plates and maintained for 16 days. Viability of cortical neurons was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, which detects mitochondrial dehydrogenase activity, as described previously (Hashimoto et al., 2002). Neuroprotection is expressed as a percentage of maximal neuroprotection, using values derived from glutamate-treated

cells and the untreated control as the reference. Cell viability is expressed as a percentage of the untreated control. Results are means±SEM of viability measurements from 5–6 cultures. One-way ANOVA was used for the statistical analysis, and significant differences in cell viability were determined by post hoc comparisons of means using Bonferroni post hoc test.

2.4. Measurement of BDNF content

Cells on a 6-well plate were collected with 250-μl of ELISA buffer (50 mM, Tris-HCl, pH 7.5, 300 mM NaCl, 0.3% Triton-X100, 10 μg/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM, benzetonium-Cl, and 1 mM benzamidine-HCl). Samples were homogenized in an ice-bath and the homogenates were sonicated five times for 5 s each and stored at −80 °C. Measurement of BDNF content by two-site ELISA was essentially performed as described by Nawa et al. (1995). Briefly, 96-well microtitre plates were precoated with turkey anti-BDNF antibody (diluted with 0.1M Tris-HCl pH 9.0) at 4 °C overnight. Supernatants of centrifuged samples in duplicate or BDNF standards in triplicate (1–300 pg) in a total volume of 90-μl were added to each well of the plates and incubated at 4 °C overnight. After washing, biotinylated anti-BDNF antibody in ELISA buffer was added to the wells followed by incubation at 4 °C overnight. Avidin-β-galactosidase was added to the well to detect bound biotinylated secondary antibody. The fluorescence intensity was measured in a fluorometer at 364 nm excitation and 448 nm emission wavelengths after reacting with the fluorogenic reagent, 4-methyl-umbelliferyl-β-galactoside.

2.5. Immunoprecipitation and western blotting

TrkB immunoprecipitation was performed as described previously (Takei et al., 1998). Cells were lysed with radioimmune buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml PMSF, and 1 mM sodium orthovanadate). The lysates were cleared by centrifugation with an excess amount of protein-G Sepharose (Amersham Pharmacia Biotech, Arlington Heights, IL). The supernatants were incubated with anti-TrkB antibody (polyclonal, Santa Cruze Biotechnology, Santa Cruz, CA), and the immune complexes were collected after incubating with protein-G Sepharose. The immunoprecipitates were washed, and then dissolved in the sample buffer for SDS-polyacrylamide gel electrophoresis and Western blotting, as described previously (Hashimoto et al., 2001). Separated proteins were transferred onto a polyvinylidene fluoride membrane, which was incubated with either anti-TrkB antibody (monoclonal; BD Transduction Laboratories, San Diego, CA) or anti-phospho-TrkB antibody (polyclonal against phosphoTyr490; Cell Signaling Technology, Beverly, MA) as the first antibody, washed, and then exposed to the secondary antibody, horseradish peroxidase-conjugated anti mouse or anti rabbit IgG (Amersham Pharmacia Biotech). Immunoreactivie protein bands were visualized by ECL detection and quantified using a CCD camera (Sierra Scientific) and Macintosh NIH Image 1.6 software. Statistical differences between untreated and lithium-treated samples were analyzed by Student's *t* test.

3. Results

3.1. Neuroprotective effects of lithium and BDNF against glutamate excitotoxicity in cortical neurons

Cerebral cortical cultures after 15 days in vitro were exposed to glutamate (8 μM) for 24 h with or without lithium or BDNF pretreatment and then stained with MTT prior to morphological assessments (Fig. 1A–D). These cortical neurons extended long neurites (Fig. 1A), and glutamate treatment resulted in cell death and an extensive loss of neurites (Fig. 1B). This glutamate-induced excitotoxicity was largely prevented by pretreatment with either 1 mM LiCl for 6 days or 50 ng/ml of BDNF for 1 day (Figs. 1C and D). Dose and time-dependent neuroprotection by lithium in cortical cultures has been established previously (Hashimoto et al., 2002). To characterize the neuroprotective effects of BDNF in cortical cultures, dose and time-dependence studies were performed and cell survivals were quantified by MTT assays. BDNF neuroprotection was significant in the range of 2–400 ng/ml and maximum neuroprotection was observed at 50 ng/ml (Fig. 1E). Neuroprotection required BDNF pretreatment for more than 24 and less than 72 h but the maximal effect was observed with a 24 h pretreatment (Fig. 1F).

3.2. Roles of the BDNF/TrkB pathway in lithium neuroprotection

To investigate the mechanisms underlying neuroprotective actions of lithium and BDNF, the effects of K252a, a Trk inhibitor, were studied (Fig. 2). Exposure to 8 μM glutamate decreased the viability of cortical cultures by approximately 50% and this depression of cell viability was completely reversed by a 6-day lithium pretreatment. The neuroprotective effect of lithium was partially inhibited (52%) by the presence of 200 nM K252a during pretreatment. BDNF pretreatment provided partial protection against glutamate excitotoxicity and this neuroprotection was completely blocked by K252a. Treatment with lithium, BDNF or K252a alone did not affect cell viability. Additionally, lithium neuroprotection was blocked by the presence of a BDNF neutralizing

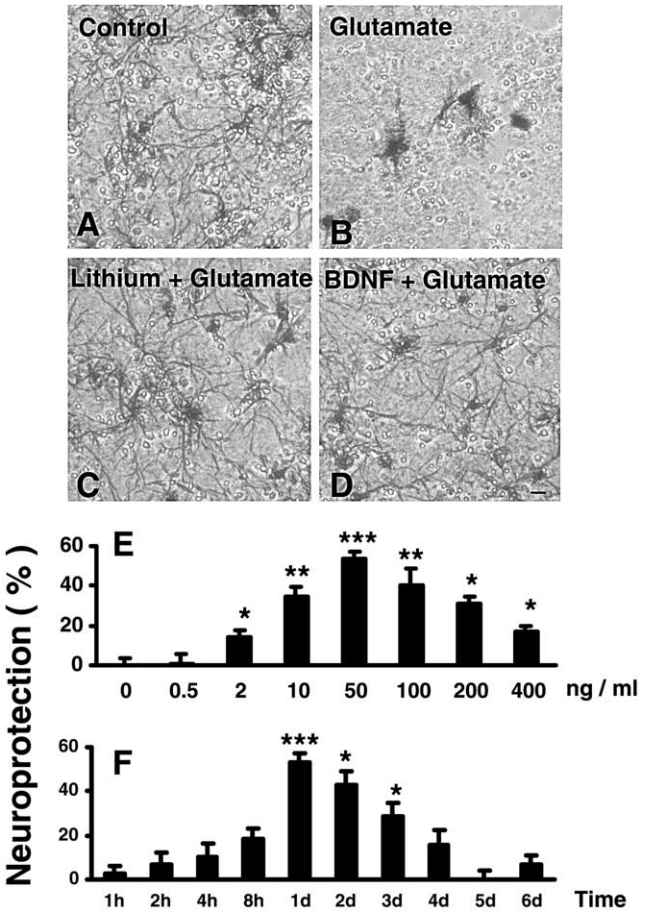

Fig. 1. Neuroprotective effects of lithium and BDNF against glutamate excitotoxicity in rat cortical cultures. (A–D) Morphological studies. Cultured cortical neurons were pretreated with vehicle, LiCl (1 mM) for six days or BDNF (50 ng/ml) for 24 h prior to exposure to glutamate (8 μM) on day 15 in vitro. Cells were stained with MTT 24 h after glutamate exposure and visualized by phase-contrast microscopy. (A) Control; (B) glutamate alone; (C) lithium+glutamate; (D) BDNF+glutamate. Bar: 10 μm. (E) Concentration-dependent neuroprotection by BDNF. Cultured cortical neurons were pretreated with the indicated concentration of BDNF for 24 h prior to glutamate exposure on day 15 in vitro. (F) BDNF (50 ng/ml) was added to cultures at various times prior to glutamate exposure. Neuroprotection was determined using the MTT assay 24 h after glutamate addition. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$, compared with the results of the group treated with glutamate alone.

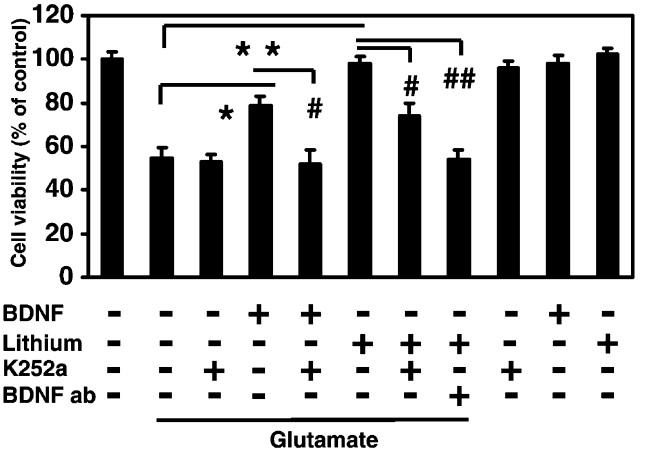

Fig. 2. K252a and BDNF neutralizing antibody inhibit lithium and BDNF-induced neuroprotection. Cells were pretreated with BDNF (50 ng/ml) for 24 h or lithium (1 mM) for six days in the presence or absence of K252a (200 nM) or BDNF neutralizing antibody (BDNFab; 10 μg/ml) and then exposed to glutamate on day 15 in vitro. Cell viability was determined using the MTT assay 24 h after glutamate addition. $*p < 0.05$; $**p < 0.01$, compared with the glutamate alone group. $\#p < 0.05$, $\#\#p < 0.01$, compared with the BDNF+glutamate or lithium+glutamate groups.

antibody. These results suggest that lithium neuroprotection might be mediated by the BDNF/TrkB signaling pathway.

To obtain further evidence for this notion, the BDNF content of cultured cortical neurons treated with lithium was measured. Intracellular BDNF protein was increased by approximately 40% following LiCl treatment for three days (ANOVA, $p < 0.05$) (Fig. 3A). Interestingly, this increase was followed by a decrease to about 65% of the untreated control on day 5, suggesting an enhanced release of BDNF from neurons into the culture medium. Since the secreted BDNF could be expected to activate TrkB, we measured the levels of TrkB phosphorylation at Tyr490 as an index of receptor activation. Treatment of cortical cultures with LiCl for 1 h to five days showed a time-dependent increase in TrkB Tyr490 phosphorylation with a 50% increase after five days of treatment (Figs. 3B and C). In contrast, protein levels of full length TrkB or truncated TrkB were unchanged during the course of lithium treatment. This increased TrkB phosporylation was suppressed by K252a (data not shown). Moreover, treatment with vehicle did not alter the levels of either total TrkB or TrkB Tyr490 phosphorylation (data not shown).

We then used BDNF mutant mice to examine whether BDNF expression is required for the neuroprotective effect of lithium. Cortical neuronal cultures were prepared from wild type (+/+), heterozygous (−/+) and homozygous (−/−) knockout mice of the same littermates and examined for their protection by lithium from glutamate-induced excitotoxicity. While glutamate-induced excitotoxicity was completely inhibited by lithium pretreatment in wild type littermates, this lithium neuroprotection was prevented in cultures derived from homozygous or heterozygous mutant mice (Fig. 4). Treatment with lithium alone did not affect cell viability in all three cortical cultures.

4. Discussion

In this study, we show that the neuroprotective effects of lithium against glutamate-induced excitotoxicity in cortical neurons can be mimicked by BDNF. The neuroprotective actions of both lithium and BDNF are sup-

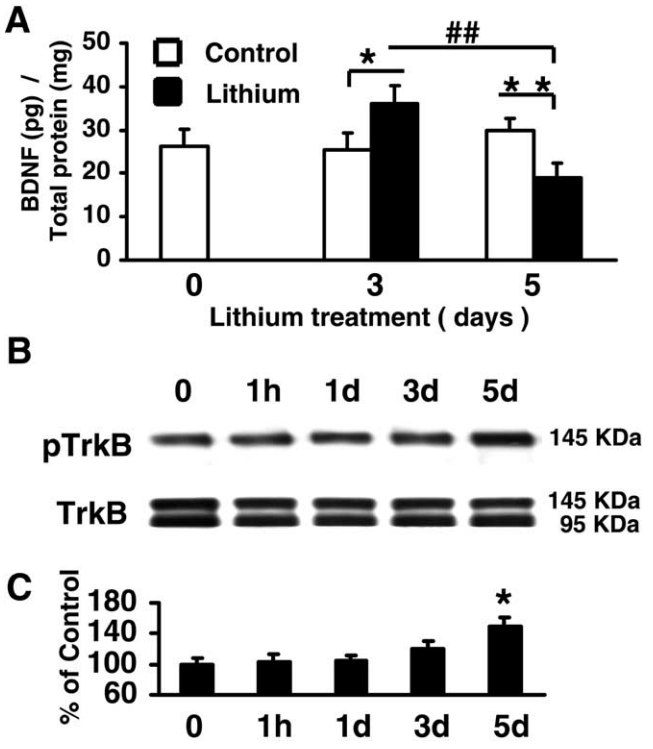

Fig. 3. Lithium regulates levels of BDNF protein and TrkB phosphorylation in cultures of neocortical neurons. (A) Measurement of BDNF protein content of cortical neurons. Cortical cultures were either untreated or treated with LiCl (1 mM) from day 9 in vitro. Cells were harvested for BDNF ELISA assay on the indicated days after treatment. $*p < 0.05$; $**p < 0.01$, compared with the corresponding untreated control. $\#\#p < 0.01$, compared with the group treated with lithium for three days. (B) Time-dependent increase of TrkB phosphorylation by lithium. Cortical cultures were either untreated or treated with LiCl (1 mM) for the indicated times. All cultures were harvested on day 15 in vitro for western blotting for total TrkB and phospho-Tyr490 TrkB. For analysis of levels of phospho-TrkB, western blotting was performed after immunoprecipitation by anti-TrkB antibody. Upper panel, phospho-TrkB immunoblots. Lower panel, TrkB immunoblots. The 145 and 95 KDa bands represent full length and truncated TrkB, respectively. The immunoblots shown are representative of three independent experiments. (C) Relative immunoreactivities of phospho-TrkB. Phospho-TrkB levels were normalized to their corresponding full-length TrkB levels and expressed as percent of untreated control. Data represent means±SEM of immunoreactiviy of three independent experiments. $*p < 0.05$, compared with untreated control.

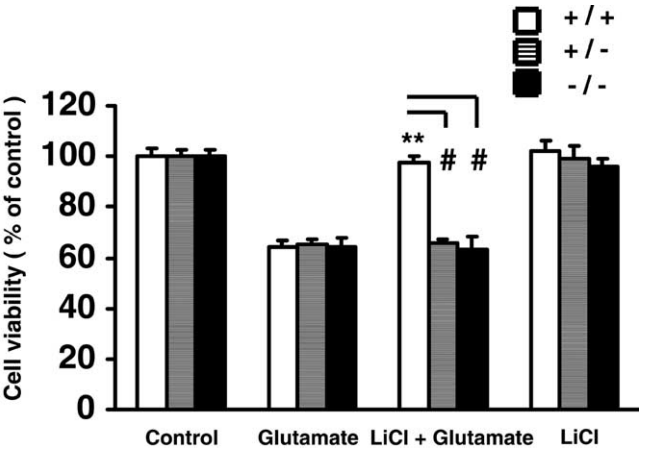

Fig. 4. Lithium fails to protect cortical neurons in BDNF mutant mice. Cortical cultures from +/+, +/− and −/− BDNF mutant mice embryos were either untreated or pretreated with LiCl (1 mM) for six days starting on day 9 in vitro and then exposed to glutamate (30 μM) on day 15 in vitro. Cell viability was determined using the MTT assay 24 h after the addition of glutamate. $**p < 0.01$, compared with the +/+ group of glutamate alone. $\#p < 0.05$, compared with the corresponding wild type group.

pressed by the Trk receptor inhibitor, K252a. Cellular levels of BDNF in cortical cultures are transiently increased after three days of lithium treatment. The decrease in intracellular BDNF on day 5 is presumably due to an enhanced release of this neurotrophin into the culture medium. Unfortunately, the concentration of BDNF in the culture medium was below the sensitivity of our ELISA assay and could not be determined. However, the kinetics of TrkB Tyr490 phosphorylation confirms our presumption of enhanced release. These results are consistent with the notion that the synthesis/release of BDNF and subsequent activation of TrkB are essential for lithium neuroprotection against glutamate excitotoxicity. The requirement of longer pretreatment time for lithium than for exogenous BDNF may reflect the time delay needed for lithium to induce a series of intracellular signaling, leading to BDNF expression and its secretion to act on TrkB receptors.

Direct evidence supporting the above notion is our observation that neuroprotective effects of lithium were completely abolished when cortical neurons were prepared from BDNF gene knockout mice. Interestingly, this complete blockade of lithium neuroprotection was shown in both BDNF homozygous (−/−) and heterozygous (+/−) knockout mice. These results indicate that a critical level of BDNF induced by lithium is essential to protect these neurons from excitotoxicity. Similar to our observations, it has been reported that BDNF (−/−) and (+/−) mice show the same degree of impairment in long-term potentiation in the hippocampus (Korte et al., 1995; Patterson et al., 1996; Pozzo-Miller et al., 1999). The failure of BDNF knockout or K252a treatment to exacerbate glutamate-induced cell death indicates that the absence of endogenous BDNF and TrkB activity does not contribute to glutamate excitotoxicity.

Enhanced phosphorylation of TrkB Tyr490 activates two cell-survival pathways: the PI 3-kinase/Akt and MAPK/ERK pathways (Patapoutian and Reichardt, 2001; Takei et al., 1999). The former pathway leads to Akt-dependent phosphorylation and hence inactivation of pro-apoptotic proteins such as BAD (Datta et al., 1997), caspase-9 (Cardone et al., 1998) and glycogen synthase kinase-3 (Moule et al., 1997) and of cytoprotective factors such as CREB (Du and Montminy, 1998). Phosphorylation and activation of CREB can also be

achieved by the MAPK/ERK pathway through ribosomal S6 kinase 2 (Xing et al., 1996). It has been shown that CREB activation is essential for the survival of cerebellar granule cells and sympathetic neurons (Bonni et al., 1999; Riccio et al., 1999) and this action is likely due to its ability to stimulate the expression of cytoprotective proteins such as BDNF and Bcl-2 (Finkbeiner, 2000). Thus, the long-term (5–7 days) effects of lithium on Akt phosphoryation and activation (Chalecka-Franaszek and Chuang, 1999), ERK phosphorylation (Kopnisky et al., 2002), CREB phosphorylation (Ozaki and Chuang, 1997) and Bcl-2 upregulation (Chen and Chuang, 1999) in cultured CNS neurons are likely the results of lithium-induced BDNF induction/TrkB activation. Lithium-induced decrease in p53 mRNA and protein (Chen and Chuang, 1999; Lu et al., 1999) could also be related to an Akt-dependent depression of NFkB activity, which is required for the transcription of p53 mRNA (Beraud et al., 1999).

Environmental stressors such as immobilization, which have been linked to depression, decrease BDNF mRNA (Duman et al., 1999; Smith et al., 1995). Chronic treatment of animals with antidepressants upregulates mRNA of BDNF and TrkB (Nibuya et al., 1995) and administration of BDNF alone shows antidepressant activity in rodents (Duman et al., 1999). Recently, chronic lithium treatment was reported to increase the levels of BDNF in the cortex and hippocampus of the rat brain (Fukumoto et al., 2001). The lithium-induced increase in BDNF expression and activation of TrkB reported in the present study could therefore be useful for the understanding of the antidepressant and/or antimanic efficacy of this drug in the treatment for bipolar mood disorder. Moreover, the ability of lithium to activate BDNF/TrkB signaling and protect neurons from excitotoxicity raises the intriguing possibility that lithium might be used to reduce brain damage in patients with neurodegenerative disorders.

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