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Neuroprotection of Lithium is Associated with Inhibition of Bax Expression and Caspase 8 Activation

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Neuroprotective properties of lithium were investigated by using *in vivo* NMDA excitotoxicity model. The appearance of TUNEL positive cells was prominent within 24 h of NMDA (70 mg/kg, i.p.) injection in the regions of the cortex, hippocampal formation, and thalamus of mouse cerebrum. NMDA treatment resulted in the extensive enhancement of Bax immunoreactivity in the cortical and hippocampal regions. NMDA also increased the immunoreactivity of caspase 8 in the similar regions of the mouse cerebrum. However, the increased immunoreactivity of Bax and caspase 8 were dramatically attenuated by chronic lithium pretreatment (lithium chloride, 300 mg/kg/d, i.p. for 7~10 days). At the same time, lithium ion blocked the appearance of TUNEL positive cells, and the morphological assessment indicated an effective neuroprotection by lithium against NMDA excitotoxicity. Although the exact action mechanism of lithium is not straightforward at this time, we propose that the inhibition of Bax and caspase cascade is involved in the neuroprotective action of lithium.

Key Words: Bax, Caspase 8, Lithium, NMDA, Neuronal apoptosis

INTRODUCTION

Lithium has been introduced into the treatment and prophylaxis of manic depressive illness (MDI) almost half a century ago. On the action mechanism of lithium, many researchers have already suggested inhibition of the phosphatidylinositol cycle, but recently have attracted on the novel targets of signal transduction system regulating protein kinase C (Jope, 1999; Manji & Lenox, 1999), transcription factors (Wang et al, 1997; Jope, 1999), and gene expressions (Nestler, 1998; Jope, 1999). However, the exact mechanism that underlies therapeutic actions of lithium on the MDI has not been fully elucidated.

Moreover, exciting new sets of evidence indicating that lithium has a neuroprotective effect have emerged recently (D'Mello et al, 1994; Centeno et al, 1998; Nonaka & Chuang, 1998; Nonaka et al, 1998; Manji et al, 1999). D'Mello et al (1994) have reported that lithium blocked apoptosis induced by low potassium concentration in mature cerebellar granule neuronal culture. Nonaka et al (1998) have reported that lithium exerts a protection against direct glutamate excitotoxicity by inhibition of calcium influx through NMDA receptors. Centeno et al (1998) have reported an evidence that lithium prevents apoptosis induced by cells permeable to ceramide analog, N-acetyl-D-sphingosine in cerebellar granule cell culture. Nath et al (2000) have reported that caspase activation is linked to NMDA-induced apoptosis in neonatal rat brains. Here, we supposed that neuroprotective property of lithium can be proved by examining the expression of genes related to neuronal apoptosis. In an attempt to elucidate the molecular mechanism underlying neuroprotection of lithium, we used *in vivo* NMDA excitotoxicity model, and neuronal apoptosis was evaluated following the systemic application of NMDA in young mouse cerebrum.

Bax is a proapoptotic gene that induces the leakage of cytochrome c by disrupting mitochondrial integrity following cell injuries, and activates caspases that lead to irreversible cell death (Susin et al, 1997; Kuwana et al, 1998). Caspase 8 is activated early in

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apoptotic process and is considered as an initiator cystein protease in apoptotic pathways *via* death receptors (Boldin et al, 1996; Muzino et al, 1996). In this study, we have examined the neuroprotective property of lithium by investigating the levels of Bax and caspase 8 expression against NMDA excitotoxicity.

METHODS

Materials

Lithium chloride and NMDA were purchased from Sigma Chemical Company (St. Louis, MO, USA). All other reagents were of analytical grade, and purchased from various commercial sources. Primary antibodies were obtained from Santa Cruz Biotechnology (Bax: B-9) and Calbiochem (Caspase-8: Ab-1).

Experimental protocol and preparation of brain sections

Young ICR mice weighing 10~15 g were used in this study. Experimental mice were about 2 weeks of age and included males and females. Mice were housed in cages on a constant 12 h light/12 h dark cycle and given *ad libitum* access to food and water. Experimental mice were divided into three groups: saline-treated group (control), NMDA-treated group (70 mg/kg, i.p.), and chronic LiCl-treated group (300 mg/kg, i.p.). LiCl was given once daily for 7~10 days. This treatment protocol attained serum drug concentration of 0.4~0.5 mEq/L. On average 4~6 animals were used in each group. All the experimental drugs were given in a volume of 0.1 ml/10 g body weight. All animal experiments were performed in accordance with protocols approved by the committee on Animal Care and Experimentation at School of Medicine, Keimyung University.

To harvest the whole brain the animals were deeply anesthetized with sodium pentobarbital and underwent transcardiac perfusion with heparinized (2 units/ml heparin) phosphate-buffered saline (PBS) and followed by 10% phosphate-buffered formalin (PBF). For TUNEL and immunohistochemistry, the brains were removed, postfixed for 1~2 days in 10% PBF. The fixed brain tissues were processed in an automatic tissue processor and carefully embedded in the molten paraffin in metallic blocks, covered with flexible plastic molds. Coronal sections with 7 μm thickness were made from each sample and serially mounted on 3-aminopropyltriethoxysilane (Sigma, St. Louis, MO, USA)-coated slides.

TUNEL (TdT-mediated dUTP-biotin nick end labeling) stain

Apoptotic cell death was evaluated using TUNEL method using In Situ Cell Death Detection Kit (Roche Molecular Biochemicals, Germany). In brief, brain sections were deparaffinized with xylene and hydrated in a graded series of ethanol (100, 95, 80 and 70%) and double deionized water. Sections were treated with proteinase K (20 μg/ml in 10 mM Tris-HCl, pH 7.4~8.0) for 15 min at room temperature in a humidified chamber. After three washes in PBS, specimens were incubated with TUNEL reaction mixture [terminal deoxynucleotidyl transferase (TdT) and dUTP-labeled nucleotide mixture] for 1 h at 37°C in a humidified chamber. The sections were washed in PBS for 5 min three times. Then the sections were incubated with anti-fluorescein alkaline phosphatase for 60 min at 37°C in a humidified chamber and washed in PBS for 5 min three times. Then the sections were stained with NBT/BCIP as a substrate for the alkaline phosphatase for about 10~20 min at room temperature in the dark. As a negative control, adjacent sections were processed accordingly, except that TdT was omitted from the reaction mixture. For a positive control, adjacent sections were treated with DNase.

Immunohistochemistry

Coronal sections were deparaffinized in xylene, hydrated in a graded series of ethanol (100, 95, 80 and 70%) and PBS. Immunohistochemistry was performed using the avidin-biotin complex technique with 3,3-diaminobenzidine (DAB) as the chromogen. Sections were incubated for 30 min in methanol containing 0.3% H_2O_2 to block endogenous peroxidase activity. Then the tissue sections were washed in PBS, and antigen retrieval was done by microwave irradiation (370 W, 10 min in 0.01 M citrate buffer, pH 6.0). After two PBS washes, the sections were incubated overnight at 4°C with the primary antibody for either Bax or caspase 8. Immunolabeling was detected using a biotinylated universal immunoglobulins followed by visualization with an streptavidin

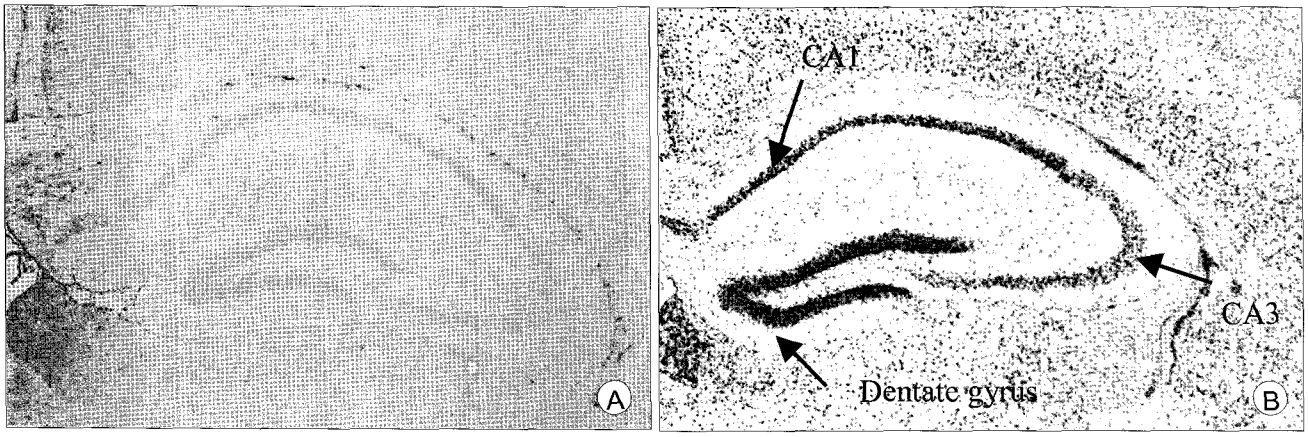

Fig. 1. Representative microphotographs of in situ apoptotic DNA fragmentation detected by TUNEL staining. (A) 1 day after NMDA injection with lithium pretreatment (300 mg/kg/d, i.p. for 7 days). (B) 1 day after NMDA injection. TUNEL positive cells were seen throughout the hippocampus and cerebral cortex by 1 day after NMDA injection (B), but lithium pretreatment completely blocked the appearance of the nuclear DNA fragmentation (A). Original magnification × 10.

peroxidase kit (DAKO, LSAB kit) and DAB staining. The sections were counterstained in hematoxylin and mounted with Canada Balsam.

RESULTS

Protection by lithium chloride on the apoptotic cell death

The nuclear DNA fragmentation was observed as early as after 6 h following NMDA administration (data not shown). Both pyknotic nuclei (Nissl stain) and DNA fragmentation (TUNEL method) were first appeared in cerebral cortex and hippocampal CA1 regions (Fig. 1). Thereafter, the staining intensity was increased and TUNEL-positive cells were observed in the areas of mouse cerebral cortex, hippocampal CA1, CA2, CA3 and dentate gyrus by 24 h after NMDA injection (Fig. 1). The brains pretreated with LiCl for 7 days completely blocked the occurrence of TUNEL-positive nuclei induced by NMDA (Fig. 1, 2).

Bax and caspase 8 expression by NMDA excitotoxicity

The brains of NMDA-treated mice had a significant increase in Bax and caspase 8 immunoreactivity in regions of retrosplenial granular cortex, dorsal auditory cortex, and the CA1 and CA3 of the hippocampal formation (Fig. 3, 4). Bax expression level

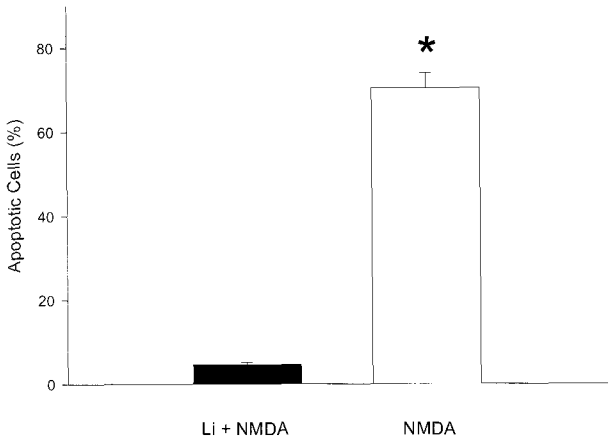

Fig. 2. Inhibition by lithium against NMDA-induced apoptosis in hippocampal formation. Apoptotic cells were counted in 12 fields per treatment and expressed as a fraction of total neuronal nuclei. Statistical analysis consisted of a Student's t test. Data are given as mean± SE of three independent experiments. *$P<0.01$.

was determined in the granular layer of dentate gyrus, hippocampal CA1, CA2, CA3 region, lateral and medial habenular nuclei, reticular thalamic nuclei, and medial globus pallidus (Fig. 3), which correlated with the increased expression of caspase 8 (Fig. 4).

Inhibition of Bax and caspase 8 expression by chronic lithium treatment

The chronic lithium treatment markedly inhibited

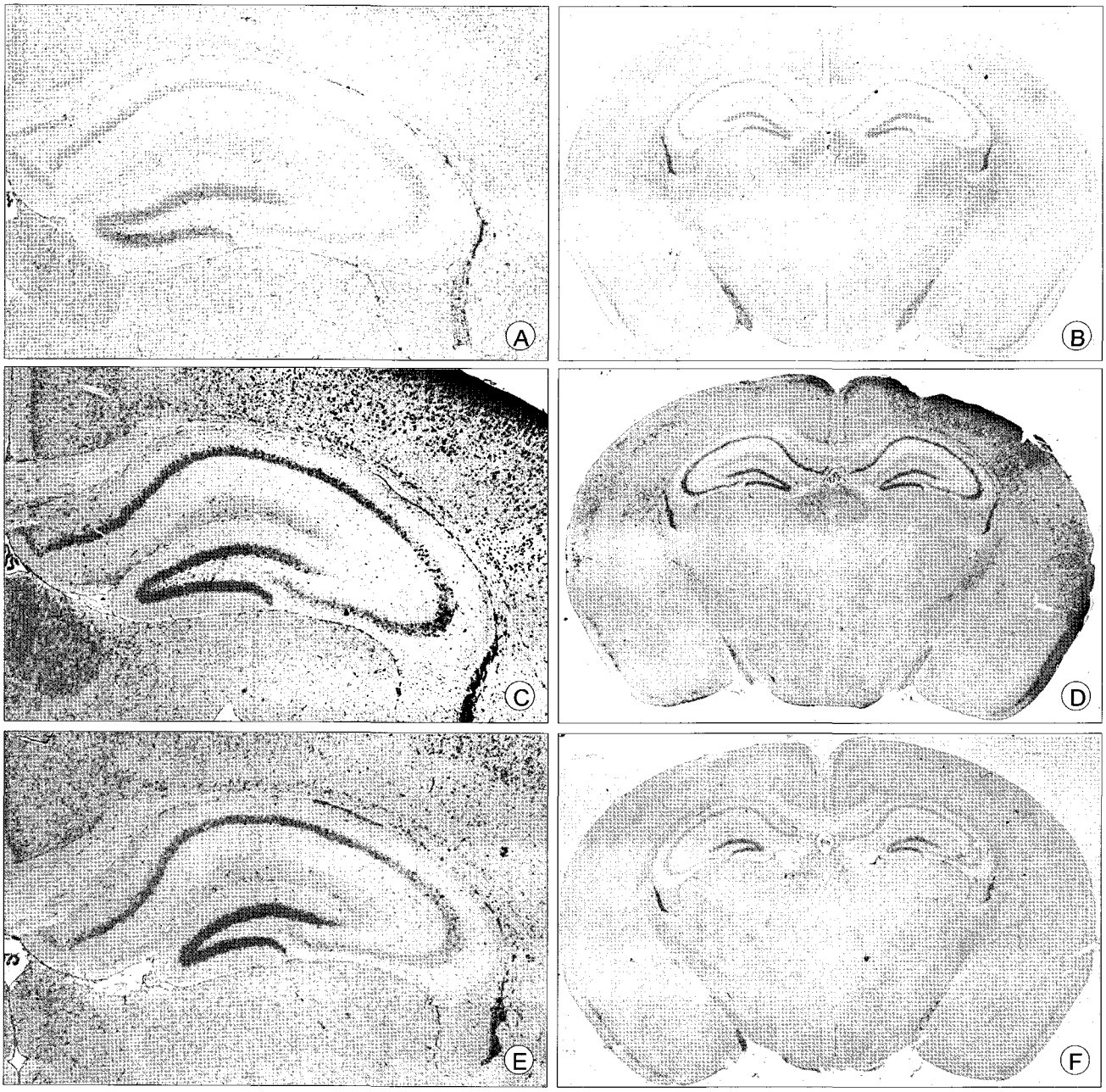

Fig. 3. Representative microphotographs of mouse hippocampal formation (A, C, E) and cerebral region (B, D, F) showing the Bax immunoreactivity of normal (A, B), 1 day after NMDA injection (C, D), and 1 day after NMDA injection with lithium pretreatment (300 mg/kg/d, i.p. for 7 days) (E, F). The panels A, C, E represent original magnification of $\times 10$. The panels B, D, F represent original magnification of $\times 1.25$.

the appearance of Bax immunoreactive cells induced by NMDA (Fig. 3). At the same time the inhibition of caspase 8 expression was similar to that of Bax (Fig. 4).

DISCUSSION

Numerous *in vivo* and *vitro* studies have demonstrated that overstimulation of glutamate receptors can induce apoptotic cell death (Linnik et al, 1993; Ankarcrona et al, 1995; Bonfoco et al, 1995: Ayata

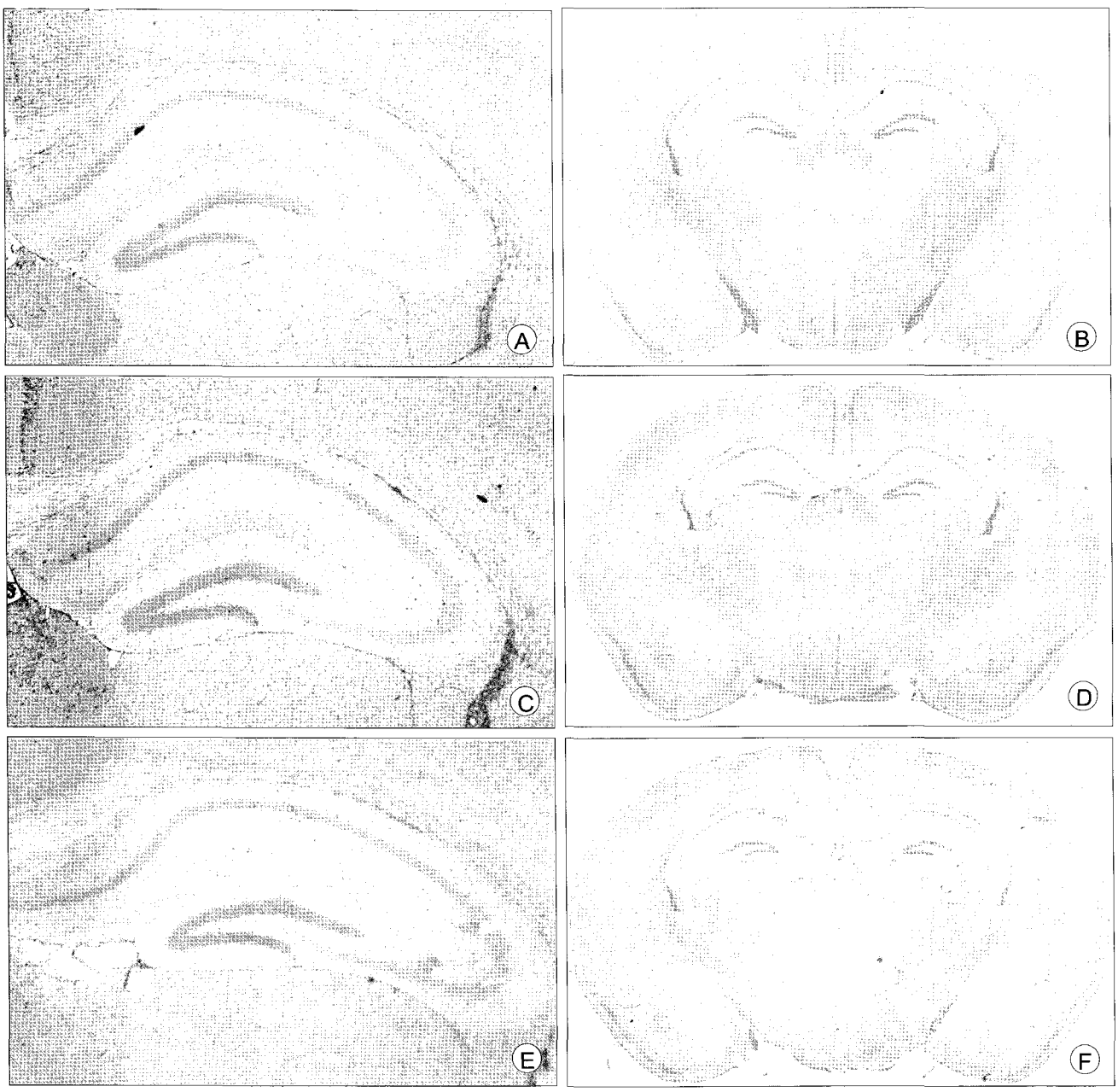

Fig. 4. Representative microphotographs of mouse hippocampal formation (A, C, E) and cerebral region (B, D, F) showing the caspase 8 immunoreactivity of normal (A, B), 1 day after NMDA injection (C, D), and 1 day after NMDA injection with lithium pretreatment (300 mg/kg/d, i.p. for 7 days) (E, F). The panels A, C, E represent original magnification of $\times 10$. The panel B, D, F represent original magnification of $\times 1.25$.

et al, 1997; Tenneti & Lipton, 2000). However, the details of apoptotic signaling pathways have not been clearly worked out in the CNS. Excitotoxic injuries result in either necrosis or apoptosis depending on the duration and intensity of insults (Ankarcrona et al, 1995; Bonfoco et al, 1995: Ayata et al, 1997).

In the present study, the extent of neuronal damage caused by the systemic injection of NMDA was evaluated by TUNEL method and Nissl stain. Nissl stain revealed the appearance of pyknotic nuclei and a decrease in Nissl bodies (data not shown). TUNEL positive cells appeared in hippocampal CA1 region as early as 6 h after NMDA injection. The number of TUNEL positive cells increased and appeared espe-

cially in the regions of cerebral cortex and hippocampal formation by 24 h of NMDA treatment. However, 7~10 days of lithium pretreatment completely blocked the NMDA-induced neuronal cell death based on the morphological assessment of apoptosis by TUNEL assay (Fig. 1, 2).

To further assess the role of chronic lithium against the excitotoxic NMDA injury, the immunoreactivity of proapoptotic gene products such as Bax and caspase 8 were examined. NMDA treatment resulted in a dramatic increase in the immunolabeling of Bax in the regions of frontal cortex, hippocampal CA1 and CA3, and dentate gyrus (Fig. 3). Also the immunolabeling of the initiator cystein protease, caspase 8 was elevated by NMDA injection in the similar regions (Fig. 4). However, 7~10 days of lithium pretreatment resulted in a dramatic inhibition of both of these apoptosis-related gene expressions (Fig. 3, 4).

Bax initiates cell death program in relation to mitochondrial dysfunction, which is a critical factor that determines the mode of apoptotic cell death (Susin et al, 1997; Jurgensmeier et al, 1998; Kuwana et al, 1998). Jurgensmeier et al (1998) have reported an evidence that Bax directly induces release of cytochrome c (Cyt c) preceding the loss of electrochemical gradient across the inner membrane of mitochondria. Cyt c in cytosol allows activation of caspases, the set of precise cell death proteases. Caspase 8 is activated at the apex of caspase pathway linked to death domain protein, FADD in TNFR1 and Fas ligand signaling with caspase 10, while caspase 3 and caspase 7 are activated at a later phase of apoptosis (Green & Reed, 1998; Thornberry & Lazebnik, 1998). Disruption of caspase 8 gene ablates neuronal cell death induced by TNF, Fas, and DR3 receptor in mouse (Varfolomeev et al, 1998). Therefore, the increase of caspase 8 expression suggests an initiation of apoptosis induced by these death receptors.

In the present study, we have shown that neuronal apoptosis induced by NMDA was protected by chronic lithium pretreatment based on Bax and caspase 8 immunoreactivity. Especially, 7~10 days of lithium treatment provided the most significant neuroprotection against NMDA excitotoxicity, which occurred at the serum drug concentration of 0.4~0.5 mEq/L. The concentration of lithium used in this study was comparable to the effective dose used in other reports, and this is about half of the therapeutic concentration used in clinical treatment of MDI. There was no significant weight loss observed during chronic lithium administration. However, acute treatment (1~5 days) of lithium did not inhibit the neuronal injury caused by NMDA in this *in vivo* model. These findings are compatible with earlier reports describing the neuroprotective properties of this monovalent cation by using cell culture systems (Centeno et al, 1998; Nonaka & Chuang, 1998; Nonaka et al, 1998). According to the results obtained in this study, we can suppose that the neuroprotective property of lithium may be associated with inhibition of Bax and activation of caspase 8 in apoptotic signaling pathways.

Excessive extracellular glutamate causes nerve cell death in stroke or trauma *via* NMDA receptors (Rothman & Olney, 1986; Choi 1987). Also excitotoxicity is mediated primarily by the entry of calcium ions through NMDA receptors (Choi 1987; Connor et al, 1987; Hyrc et al, 1997). NMDA receptors mediate neurotoxicity by generating the overload of Ca^{2+}, nitric oxide, and reactive oxygen species. NMDA-mediated neurotoxicity is thought to be a common cause in a variety of acute and chronic neurologic disorders including focal ischemia, epilepsy, Huntington's disease, Alzheimer's disease, amyotrophic lateral sclerosis, and other neurodegenerative diseases (Peng et al, 1998; Luetjens et al, 2000). Recently, the postmortem studies that demonstrate reduction of neuronal and glial cell number in prefrontal cortex of MDI patients were reported (Rajkowska et al, 1999; Rajkowska, 2000). In this regard, the antiapoptotic property of lithium shown in this study may be relevant to the therapeutic aspects of lithium in the long term treatment of mood disorders. The fact that lithium protects the NMDA-induced excitotoxicity could provide molecular and cellular evidence which can be extended into therapeutic applications of lithium in the treatment of various neurodegenerative disorders in future.

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