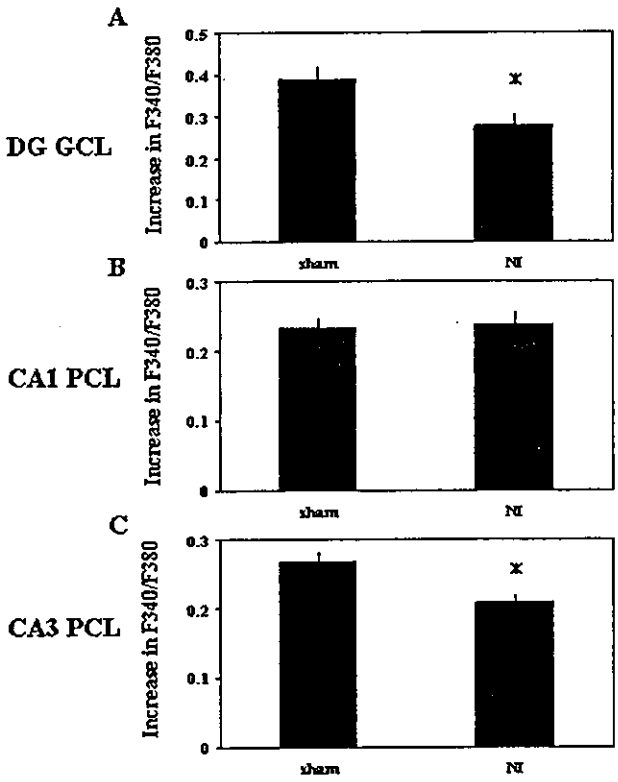

Fig. 6. Effect of NI on 100 μM NA-stimulated $[Ca^{2+}]_i$ increase. The 100 μM NA-mediated $[Ca^{2+}]_i$ increase was significantly downregulated by NI in both the DG granule cell (A) and the CA3 pyramidal cell layers (C) ($P < 0.05$), but not in the CA1 pyramidal cell layer (B). The results are the means ± SEM (n = 6).

slices per individual rat were averaged and six rats were used per group.

Characterization of the $[Ca^{2+}]_i$ increment in NI-treated rats

It is plausible that NI may regulate α_1-, α_2-, and β-adrenergic receptor pathways independently. Therefore, to examine this we measured the effects of 100 μM α_1-, α_2-, and β-adrenergic antagonists and agonists on $[Ca^{2+}]_i$ in hippocampal slices obtained from NI-treated rats (n = 5 in each experiment) (Fig. 7, Table II). Table II shows the F_{340}/F_{380} values as well as the ratio to NA. The suppression of NA-stimulated $[Ca^{2+}]_i$ increases by the adrenergic antagonists was similar in both NI-treated and sham-treated hippocampal slices (Fig. 7, Table II). Likewise, the effects of the three adrenergic agonists on the $[Ca^{2+}]_i$ increases in the hippocampal slices were similar in sham-treated and NI rats (Fig. 7, Table II).

Influence of EE on the NI-induced attenuation in $[Ca^{2+}]_i$ increment

We investigated whether the effect of NI was alleviated by EE in the present study. The NI-induced decrease in the 100 μM NA-stimulated $[Ca^{2+}]_i$ increase was alleviated by EE in the CA3 pyramidal cell layer (NI + EE, F_{340}/F_{380} ratio: 0.260 ± 0.022, NI, F_{340}/F_{380} ratio: 0.212 ± 0.011; Fig. 8C), but not in the DG granule cell layer (NI + EE, F_{340}/F_{380} ratio: 0.280 ± 0.015, NI, F_{340}/F_{380} ratio: 0.279 ± 0.022; Fig. 8A). The NA-stimulated increase in $[Ca^{2+}]_i$ in the CA1 pyramidal cell layer was not changed by NI or by EE (Fig. 8B). We also analyzed other parameters such as the latency to the $[Ca^{2+}]_i$ peak and the slope (increase/latency) (Table I). The results represent the mean values and SEMs obtained from six rats.

Influence of EE on NA-stimulated $[Ca^{2+}]_i$ increase

In rats with EE the peak increases in NA-stimulated $[Ca^{2+}]_i$ were 0.406 ± 0.030 in the DG granule cell layer and 0.280 ± 0.015 in the CA3 pyramidal cell layer (n = 6 in each group). No significant difference was detected between the sham-treated rats and EE-treated rats.

DISCUSSION

Mechanisms by which noradrenaline increases $[Ca^{2+}]_i$

The present study using AR antagonists and agonists indicates that the NA-stimulated increase in $[Ca^{2+}]_i$ was mediated predominantly by α_1-receptors, but α_2- and β-ARs contributed to the increase in $[Ca^{2+}]_i$ as well. It is reported that the α_1-AR-mediated increase in $[Ca^{2+}]_i$ is due to enhanced Ca^{2+} release from intracellular compartments, such as the endoplasmic reticulum, as well as to enhanced Ca^{2+} influx from extracellular sources (Minneman, 1988). It is well known that the stimulation of α_1-ARs induces the activation of phospholipase C (PLC) via G-proteins, and subsequently produces inositol 1,4,5-triphosphate (IP_3) through the hydrolysis of phosphatidylinositol 4,5-bisphosphate by PLC (Minneman and Esbenshade, 1994). IP_3 can bind to its specific receptor on the endoplasmic reticulum and increase Ca^{2+} flux through the IP_3R channel (Berridge, 1993). In addition, the influx of extracellular Ca^{2+} may be through voltage-dependent and voltage-independent calcium channels (Minneman, 1988). Taken together, it is conceivable that two different signal transduction mechanisms through the α_1-ARs are involved in the increase in $[Ca^{2+}]_i$ mediated by NA.

It was also evident in this study that α_2- and β-ARs in the rat hippocampus were stimulated by NA application. Facilitation of the opening of VOCCs (voltage-operated Ca^{2+} channels) is suggested to be induced by activation of α_2-ARs in porcine uterine longitudinal muscle (Kitazawa et al., 2000) and by β-ARs in cultured rat astrocytes (MacVicar and Tse, 1988). In addition, it is reported that cyclic-AMP-dependent phosphorylation of IP_3 receptors increases $[Ca^{2+}]_i$ in response to the activation of α_1-ARs in lipid vesicles

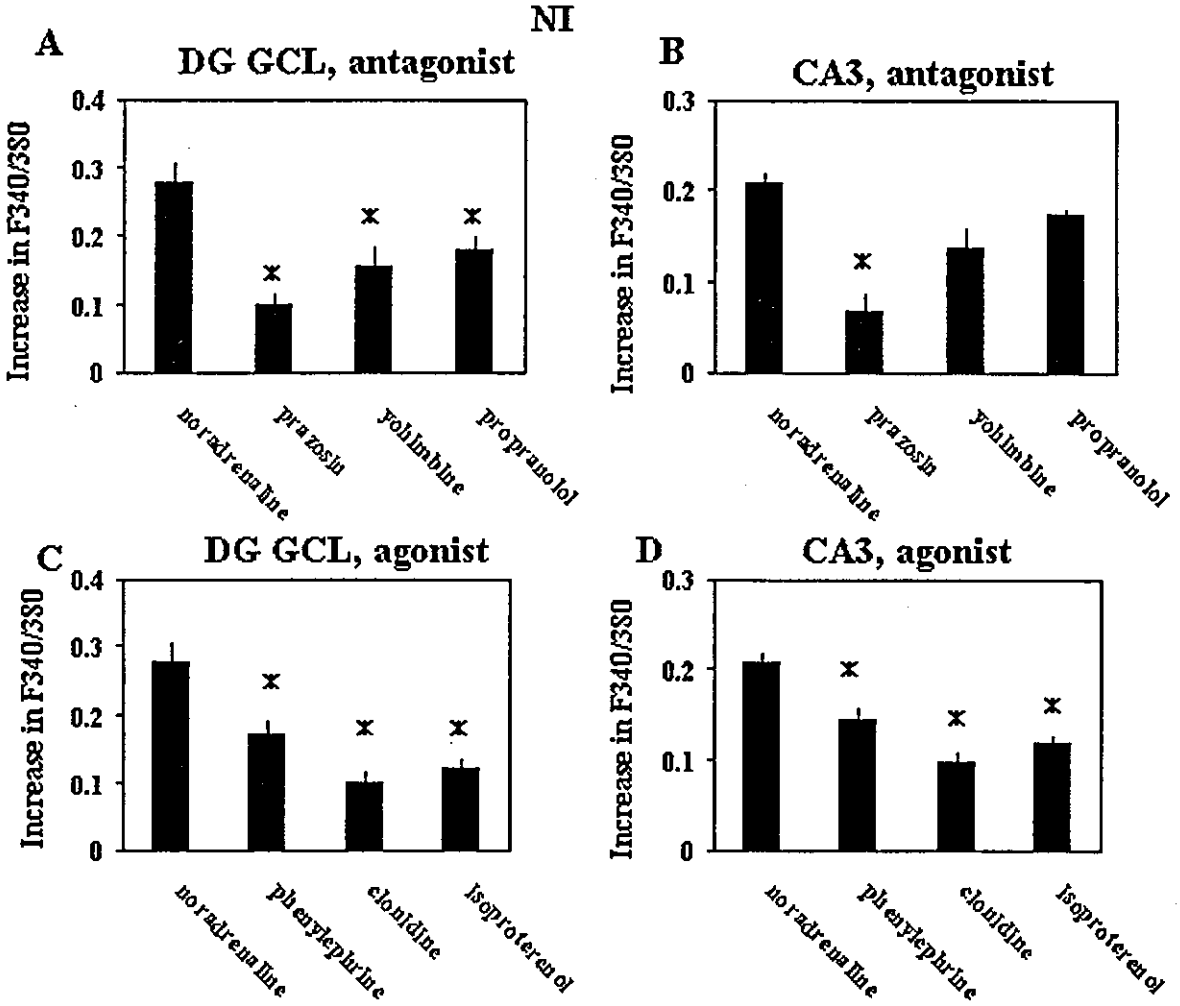

Fig. 7. Effects of adrenergic antagonists and agonists on the $[Ca^{2+}]_i$ responses in the DG granule cell (GCL) (A,C) and the CA3 pyramidal cell layers (CA3) (B,D) of NI-treated rats. The 100 μM NA-induced $[Ca^{2+}]_i$ increase was markedly reduced by 100 μM prazosin, and partially reduced by 100 μM yohimbine and 100 μM propranolol in each cell layer (A,B). 100 μM phenylephrine markedly increased $[Ca^{2+}]_i$ in both the DG granule cell (C) and CA3 pyramidal cell layers (D) while 100 μM clonidine and 100 μM isoproterenol had less of an effect (C,D). Note that the ratios of each agent to NA are similar to those shown in Figure 4. The results are the means ± SEM (n = 5).

from mouse cerebellum (Nakade et al., 1994). Thus, further study will be necessary to determine whether the $[Ca^{2+}]_i$ increase mediated by NA is derived from extracellular Ca^{2+} or from intracellular Ca^{2+} storage sites.

Effect of NI on $[Ca^{2+}]_i$

In the present study, we found that NA-stimulated $[Ca^{2+}]_i$ induction was downregulated by NI in both the DG granule cell and CA3 pyramidal cell layers. To clarify the difference between sham-treated and NI-treated rats in terms of NA-induced $[Ca^{2+}]_i$ movement, we characterized the NA-induced $[Ca^{2+}]_i$ increment in both rat groups. The α_1-, α_2-, and β-adrenergic receptor-mediated $[Ca^{2+}]_i$ increases were suppressed to a similar degree in both the NI- and sham-treated slices, so it is clear that NI did not suppress α_1-, α_2-, and β-adrenergic receptor pathways independently.

Possible mechanisms of $[Ca^{2+}]_i$ increment suppression via adrenaline receptor function

A couple of studies have shown that neonatal isolation did not alter the total number of cells in the dentate gyrus granule cell and the CA3 pyramidal cell layers (Huang et al., 2002; Huot et al., 2002), so the reduction in NA-stimulated $[Ca^{2+}]_i$ in the neonatal isolated hippocampi was not considered to be caused by a reduction in the total number of cells. It is possible that suppression of signal transduction pathways, such as Ca^{2+} influx through VOCCs or IP_3 receptors on the endoplasmic reticulum may be involved in the downregulation of NA-stimulated $[Ca^{2+}]_i$ induction in re-

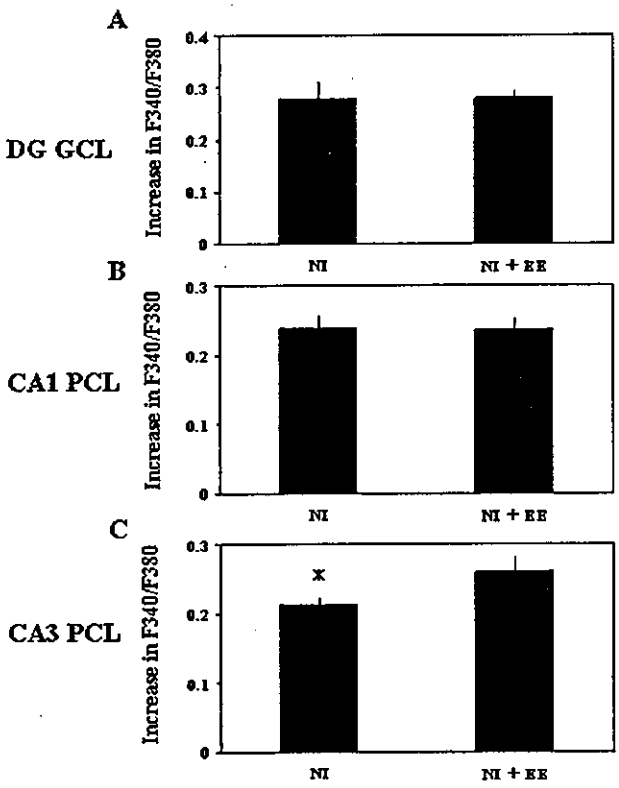

Fig. 8. Influence of EE on the downregulation of NA-stimulated $[Ca^{2+}]_i$ increment induced by NI. The downregulation in NA-stimulated elevation in $[Ca^{2+}]_i$ by NI was alleviated in response to EE in the CA3 pyramidal cell layer (C), but not in the DG granule cell layer (A). The NA-stimulated increase in $[Ca^{2+}]_i$ in the CA1 pyramidal cell layer was not affected by EE (B). The results represent the mean values and SEM (n = 6).

sponse to NI. Therefore, further studies examining the involvement of other pathways are required.

Significance of the decreased NA-induced $[Ca^{2+}]_i$ increment in NI-treated rat hippocampus

The decreased elevation in $[Ca^{2+}]_i$ might lead to a reduction in the phosphorylation of calcium/calmodulin-dependent protein kinase (CaMK) II and cAMP-responsive element binding protein (CREB), which are involved in the alteration of brain functions such as memory formation (Kida et al., 2002; Silva et al., 1992a,b). In addition, it is reported that NA promotes long-term potentiation in the rat hippocampus and plays an essential role in memory consolidation (Izumi and Zorumski, 1999; Kobayashi and Yasoshima, 2001). Taken together, it is plausible that the downregulation in NA-stimulated $[Ca^{2+}]_i$ induction in the hippocampus by NI may, at least in part, be involved in the impairment of memory function. In fact, Huot et al. (2002) reported that NI induced the impairment in spatial learning. On the other hand, it is well known that N-methyl-D-aspartate (NMDA) receptors regulate the levels of $[Ca^{2+}]_i$ essential for the induction of long-term potentiation (LTP). Therefore, it will be necessary to investigate whether NI alters $[Ca^{2+}]_i$ via NMDA receptors.

Effect of EE on the attenuated NA-induced increase in $[Ca^{2+}]_i$

Francis et al. (2002) demonstrated that EE alleviated the effects of maternal separation on both the HPA and the behavioral responses to stress. In this context, we also examined whether EE modulated the downregulation by NI of the NA-stimulated $[Ca^{2+}]_i$ increase in the CA3 pyramidal cell and DG granule cell layers. The downregulation in the NA-stimulated increase in $[Ca^{2+}]_i$ was alleviated by EE in the CA3 pyramidal cell layer, but not in the DG granule cell layer.

In published studies, the concentrations of antagonists used were typically less than 1 mM (Kobayashi et al., 1999; Okamoto et al., 1995). However, it is possible that the 100 μM antagonist concentrations used in the current study might have been insufficient to fully inhibit the ARs for the purpose of investigating the mechanisms of NA-induced $[Ca^{2+}]_i$ increases.

Effect of raising environment on the NA-induced $[Ca^{2+}]_i$ and the region-dependency of hippocampus

The NA-stimulated $[Ca^{2+}]_i$ increase was attenuated by NI in the DG granule cell and CA3 pyramidal cell layers. This downregulation was alleviated by EE in the CA3 pyramidal cell layer, but not in the DG granule cell layer. The influences of neonatal isolation and environmental enrichment are region-dependent. The mechanisms of the region-dependent influences might be associated with the different distributions of various types of cells, such as excitatory and inhibitory neurons and glial cells, since the activation of noradrenergic receptors also affects $[Ca^{2+}]_i$ mobilization in those cells. In order to elucidate a more precise mechanism, further studies are required.

In summary, whereas NA-stimulated $[Ca^{2+}]_i$ increment was downregulated by NI in both the DG granule cell and CA3 pyramidal cell layers, the administration of EE following NI alleviated the downregulation only in the CA3 pyramidal cell layer. These findings suggest that raising environments, such as NI and EE, may modulate hippocampal function by altering adrenergic receptor-mediated signal transduction during adolescence.

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β-Estradiol, Dehydroepiandrosterone, and Dehydroepiandrosterone Sulfate Protect against *N*-Methyl-D-aspartate-Induced Neurotoxicity in Rat Hippocampal Neurons by Different Mechanisms

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ABSTRACT

We examined neuroprotective effects of β-estradiol, dehydroepiandrosterone (DHEA), and dehydroepiandrosterone sulfate (DHEA-S) against *N*-methyl-D-aspartate (NMDA)-induced neurotoxicity in primary cultured rat hippocampal neurons. All three steroids demonstrated neuroprotective effects. Time-course studies revealed that steroid cotreatment for only 15 min at the same time as exposure to NMDA, but neither pretreatment nor addition of steroids for 24 h after NMDA-mediated neuroprotective effects. This indicates that short-term actions of these steroids are critical for this process. Acute treatment with β-estradiol dose dependently inhibited NMDA-induced intracellular Ca^{2+} increases, which strongly correlated with its neuroprotective effect via L-type voltage-gated calcium channels. Acute treatment with DHEA, but not with DHEA-S, significantly inhibited nitric oxide (NO) production and Ca^{2+}-sensitive NO synthase (NOS) activity caused by NMDA stimulation. An NOS inhibitor, N^G-monomethyl-L-arginine acetate was also protective against NMDA-induced neurotoxicity. These data indicate that β-estradiol may exert neuroprotective effects mainly by reducing Ca^{2+} increases but that DHEA may act by inhibiting NOS activity. Treatment with the σ-1 receptor (Sig-1R) antagonists rimcazole or BD1063 (1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride) partially, but significantly, reversed the neuroprotective effect of DHEA-S against NMDA-induced neurotoxicity, whereas muscimol, a GABA-A-receptor agonist, did not. This suggests that the neuroprotective effect of DHEA-S may be mediated via Sig-1R, at least in part. Together, our data suggest that the neurosteroid family members β-estradiol, DHEA, and DHEA-S exert neuroprotective effects through different nongenomic mechanisms.

Stress induces an elevation of excitatory amino acids in the brain, including in the prefrontal cortex, hippocampus, and basal ganglia (Moghaddam, 1993). Excitatory amino acids released by stress in the hippocampus are thought to be involved in a number of important mechanisms of neuronal cell loss and atrophy, which may occur in people with stress disorders such as recurrent depressive illness (Sheline et al., 1996). As for the mechanisms responsible for hippocampal atrophy, recent animal studies have shown that the *N*-methyl-D-aspartate receptor (NMDA-R)-mediated pathway is the most important, because NMDA-R blockade is effective in preventing stress-induced hippocampal atrophy (McEwen and Magarinos, 1997). It is established that stimulation of NMDA-R induces an influx of Ca^{2+}, consequently, activating nitric-oxide synthase (NOS) and resulting in an increase of nitric oxide (NO) release (Lipton et al., 1994). The NMDA-R-mediated mobilization of intracellular Ca^{2+} and NO has already been reported to be important for triggering NMDA-induced neurotoxicity (Dawson et al., 1991).

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ABBREVIATIONS: NMDA-R, *N*-methyl-D-aspartate receptor; NOS, nitric-oxide synthase; NO, nitric oxide; NMDA, *N*-methyl-D-aspartate; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate; CNS, central nervous system; GABA-A-R, γ-aminobutyric acid type A receptor; Sig-1R, σ-1 receptor; Sig-2R, σ-2 receptor; L-NMMA, N^G-monomethyl-L-arginine acetate; BD1063, 1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride; MK-801, (−)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; DMSO, dimethyl sulfoxide; SNP, sodium nitroprusside; BSS, balanced salt solution; WST-8, tetrazolium salt; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; ANOVA, analysis of variance; VGCC, voltage-gated calcium channel; nNOS, neuronal nitric-oxide synthase; eNOS, endothelial nitric-oxide synthase; PKC, protein kinase C.

β-Estradiol, dehydroepiandrosterone (DHEA), and dehydroepiandrosterone sulfate (DHEA-S), the major precursors of β-estradiol, are members of neurosteroid families produced in the central nervous system (CNS) (MacLusky et al., 1994). Recent animal studies have demonstrated that neurosteroids exert several important physiological functions, such as increasing dendric spine density, changing electrical activity (Moss et al., 1997) and preventing neuronal death in the CNS (Singer et al., 1996; Weaver et al., 1997; Kimonides et al., 1998). Some mechanisms underlying the neuroprotective effects of estradiol involve the classical nuclear estrogen receptors (Singer et al., 1996). On the other hand, there is increasing evidence suggesting that estradiol also has short-term and nongenomic actions, such as changing electrical excitability, synaptic functioning, and morphological features (Moss et al., 1997), that are not mediated via nuclear estrogen receptors. Moreover, neurosteroids, including DHEA and DHEA-S, are reported to have short-term actions mediated by NMDA-R, γ-aminobutyric acid type A receptors (GABA-A-R) or σ-1 receptors (Sig-1R) (Majewska et al., 1990; Monnet et al., 1995; Kurata et al., 2001). Although a large number of studies have been made carried out on neurosteroids, mechanisms of neuroprotection remain unclear.

In this context, we investigated neuroprotective effects of these steroids in cultured hippocampal neurons. We also examined the effects of β-estradiol, DHEA, and DHEA-S on NMDA-R-mediated Ca^{2+}/NO signaling pathway and relationships with GABA-A-R and Sig-1R to better elucidate mechanisms of neuroprotection.

Materials and Methods

Materials. NMDA, N^G-monomethyl-L-arginine acetate (L-NMMA), and 1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride (BD1063) were obtained from Tocris Cookson Inc. (Bristol, UK); β-estradiol, DHEA, DHEA-S, muscimol, rimcazole, poly-L-lysine, deoxyribonuclease, and nifedipine were from Sigma-Aldrich (St. Louis, MO); MK-801 was from Sigma/RBI (Natick, MA); HEPES, BAPTA, fura-2 acetoxymethy ester, cell counting kit-8, and the $NO_2/_3$ assay kit were from Dojindo (Kumamoto, Japan); the NOS assay kit was from Calbiochem (Darmstadt, Germany); trypsin, B27, and N2 were from Invitrogen (Carlsbad, CA); DMSO was from Wako Pure Chemicals (Osaka, Japan); penicillin and streptomycin were from Meiji Seika, Ltd. (Tokyo, Japan); and sodium nitroprusside (SNP) was from Katayama Chemical (Osaka, Japan). Solutions of β-estradiol, DHEA, and DHEA-S were prepared in 0.5% DMSO. This concentration of DMSO was able to dissolve β-estradiol at a highest concentration of 60 μM.

Cell Culture. Primary cultures were prepared as follows, according to the Guiding Principles on Animal Experimentation in Research Facilities for Laboratory Animal Science (Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan), for the care and use of laboratory animals. Wistar rat embryos were removed from the mother at embryonic day 19 under anesthesia. Hippocampal tissue was dissected out and incubated in 0.25% trypsin and 0.02% deoxyribonuclease I for 25 min at 37°C on a shaker, followed by inactivation of the enzymes with fetal bovine serum. The hippocampal cells were mechanically dispersed by pipetting and rinsed twice with culture medium comprising neurobasal medium supplemented with B27 (1:50) growth medium, penicillin G (50 U/ml), and streptomycin sulfate (50 μg/ml). The cells were plated at a density of 8×10^5 /ml on poly-L-lysine-coated wells (1.77 cm^2) and 96-well plates and were maintained in culture medium under a humidified atmosphere of 10% CO_2 at 37°C. On the 8th day of culture, the medium was replaced with neurobasal medium supplemented with N2 (1:50) growth medium and maintained for 2 to 3 more days. This replacement is appropriate to elucidate effects of hormones against excitatory amino acid-induced neurotoxicity because N2 growth medium contains fewer hormones and antioxidants than B27 growth medium. On the 10th or 11th day of culture, the cells were used for the following experiments.

Measurement of NMDA-Induced Neurotoxicity. To examine the effects of β-estradiol, DHEA, and DHEA-S on NMDA-induced neurotoxicity in primary hippocampal neurons, cultured cells in 96-well plates were exposed to NMDA for 15 min, together with each steroid. The protocol for treatment with steroids is shown in Fig. 2A. In addition, washing schedules are as follows. In Fig. 2A (1, 2, and 3), after the NMDA exposure, cells were washed once with the neurobasal medium (without NMDA and steroid) and incubated for an additional period of 24 h under standard culture conditions before measurements. In Fig. 2A (2; pretreatment), after the pretreatment of steroid for 24 h, cells were washed once with neurobasal medium (without steroids) before NMDA exposure. In Fig. 2A (4; post-treatment), cells were washed once with the neurobasal medium (without steroids) after NMDA exposure and incubated with the neurobasal medium (with steroids) for an additional period of 24 h before measurements. In Fig. 2A (5; co- and post-treatment), cells were washed with the neurobasal medium (with steroids) after NMDA exposure and incubated with the neurobasal medium (with steroids) for an additional period of 24 h. Sig-1R and GABA-A-R ligands (as shown in Table 1) were added to the cultures 15 min before and during NMDA exposure. After NMDA exposure, cells were washed with neurobasal medium (with steroids, Sig-1R or GABA-A-R ligands) and incubated with neurobasal medium (with steroids, Sig-1R or GABA-A-R ligands) for an additional period of 24 h before measurements. In each experiment, just before the measurement, the cells were washed once with balanced salt solution (BSS), containing NaCl (130 mM), KCl (5.4 mM), $CaCl_2$ (1.8 mM), glucose (5.5 mM), and HEPES (20 mM), adjusted to pH 7.4 with NaOH, and replaced to BSS. Thereafter, mitochondrial dehydrogenase activity, which cleaves tetrazolium salt (WST-8), was measured to assess cell viability in a quantitative colorimetric assay. WST-8 is a water-soluble tetrazolium salt that is reduced by cellular systems coupled with NADH (Mosmann, 1983). This method is a modified MTT reduction assay. After incubation with WST-8 for 4 h, the absorbance of each well was measured at 450 nm with reference wavelength at 655 nm using a microplate reader (Micro Plate Reader; Tosho, Kanagawa, Japan).

TABLE 1

Effects of Sig-1R and GABA-A-R ligands on the neuroprotective effects of DHEA-S, β-estradiol, and DHEA against NMDA-induced neurotoxicity

All cells were exposed to 100 μM NMDA for 15 min. β-Estradiol, DHEA, and DHEA-S were added at the same time or after exposure to NMDA before measurements (the same treatment as co- + post-treatment in Fig. 2A, 5).

Treatment	Cell Viability
	%
NMDA only	34.3 ± 1.7
+ Rimcazole (1 μM)	32.8 ± 1.4
+ BD1063 (1 μM)	35.0 ± 2.1
+ Muscimol (10 μM)	32.5 ± 2.6
NMDA + DHEA-S (100 nM)	46.1 ± 3.8**
+ Rimcazole	39.0 ± 1.7*,†
+ BD1063	37.6 ± 1.3*,†
+ Muscimol	46.5 ± 3.1**
NMDA + DHEA (60 μM)	56.3 ± 1.9**
+ Rimcazole	55.6 ± 2.0**
+ BD1063	52.9 ± 3.3**
+ Muscimol	53.7 ± 3.2**
NMDA + β-Estradiol (60 μM)	48.1 ± 2.8**
+ Rimcazole	48.8 ± 2.5**
+ BD1063	47.3 ± 2.1**
+ Muscimol	47.2 ± 3.4**

* $p < 0.05$; ** $p < 0.01$ compared with NMDA only; and † $p < 0.05$ compared with NMDA + DHEA-S.

Intracellular Ca^{2+} Measurements in Single Cells. Measurements of intracellular calcium concentration ($[Ca^{2+}]_i$) by fluoromicroscopic determination of fura-2 were performed as described previously (Kurata et al., 2001). Briefly, hippocampal cells were rinsed twice with BSS and then incubated with 5 μM fura-2 acetoxymethyl ester in BSS for 60 min at 37°C. The fura-2-loaded cells were perfused with BSS, warmed to 37°C, and examined at a flow rate of 1.5 ml/min on the stage of a fluorescence microscope-videocamera system (C-2000; Hamamatsu Photonics, Hamamatsu, Japan). The fluorescence intensity of fura-2 was measured at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Fluorescence was recorded at 5-s intervals, and the ratio (340 / 380 nm) of the emitted fluorescence intensities was digitized by a color image processor (Argus 50; Hamamatsu Photonics). Ratios of emitted fluorescence were calculated using a digital fluorescence analyzer (Argus 50; Hamamatsu Photonics) and converted to $[Ca^{2+}]_i$.

Measurements of NO Production and NOS Activity. We measured NO production and NOS activity according to the instructions provided with the $NO_2/_3$ assay kit (Dojindo, Kumamoto, Japan) and the NOS assay kit (Calbiochem, Darmstadt, Germany) and determined effects of steroids thereon. Briefly, as for NO production, cultured cells in 96-well plates were exposed to 100 μM NMDA (in BSS) for 5 to 60 min (four different time points) in the presence of β-estradiol, DHEA, or DHEA-S. After exposure to NMDA, 100 μl of culture BSS was added together with 10 μl of the 0.1 U/ml nitrate reductase solution to each well. Next, 100 μl of culture BSS was reacted with an equal volume of Griess reagent (0.1% naphthylethylenediamine and 1% sulfanilamide in 5% H_3PO_4) in 96-well plates for 10 min at room temperature in the dark. The absorbance at 540 nm was determined by a microplate reader (Micro Plate Reader; Tosho). NOS activity was measured as L-arginine- and NADPH-dependent generation of nitrite (NO_2^-) and nitrate (NO_3^-), a stable oxidation product of NO. The assay was performed in the presence or absence of 100 μM NMDA, steroids, L-NMMA, and BAPTA for 15 min at room temperature in 200 μl of reaction buffer containing 20 mM Tris-HCl (pH 8.0), 1 mM NADPH, 2 mM L-arginine, and 10 μM FAD. NO_3^- was reduced to NO_2^- by incubation at 37°C for 15 min with 0.1 U/ml nitrate reductase, 0.1 mM NADPH, and 5 μM FAD. The reaction was stopped by the addition of 10 U/ml lactic dehydrogenase to destroy excess NADPH for 20 min. Then, 100 μl of Griess reagent was added for 10 min and the absorbance of each well was measured at 540 nm using a plate reader (Micro Plate Reader; Tosho). NO_2^- in the control incubation (without NADPH, L-arginine) was subtracted from experimental values.

Statistical Analysis. All experiments were conducted at least four times to ensure reproducibility. The data are presented as means ± S.E.M. In each experiment, averaged data from five to seven cells were used for analysis. Student's unpaired *t* test was used to compare between the two groups in Figs. 1B, 3B, and 4, and Table 1. Two-way analysis of variance (ANOVA) was also used in Fig. 3B. For multiple comparisons, data were analyzed by ANOVA followed by protected least-significant difference test in Figs. 1A, 2B, 3 (B and C), 5 (A and B), and 6. A P value of <0.05 was considered statistically significant. The correlation coefficient between Figs. 1A and 3B was analyzed by Pearson's correlation coefficient. The IC_{50} values were determined using regression lines of the log concentration-response curves generated by computer.

Results

Neuroprotective Effects of β-Estradiol, DHEA, and DHEA-S against NMDA-Induced Neurotoxicity. Primary cultures of hippocampal neurons were exposed to 100 μM NMDA in culture medium for 15 min, and cell viabilities were determined after 24 h by the modified MTT assay. β-estradiol and DHEA (1–60 μM, respectively) showed significant neuroprotective effects in a concentration-dependent manner (Fig. 1A). In contrast, DHEA-S showed significant neuroprotective effects at lower concentrations (10 nM–1 μM), such as a reverse U-shaped curve. Maximum protective effects were obtained at the concentration of 60 μM for both

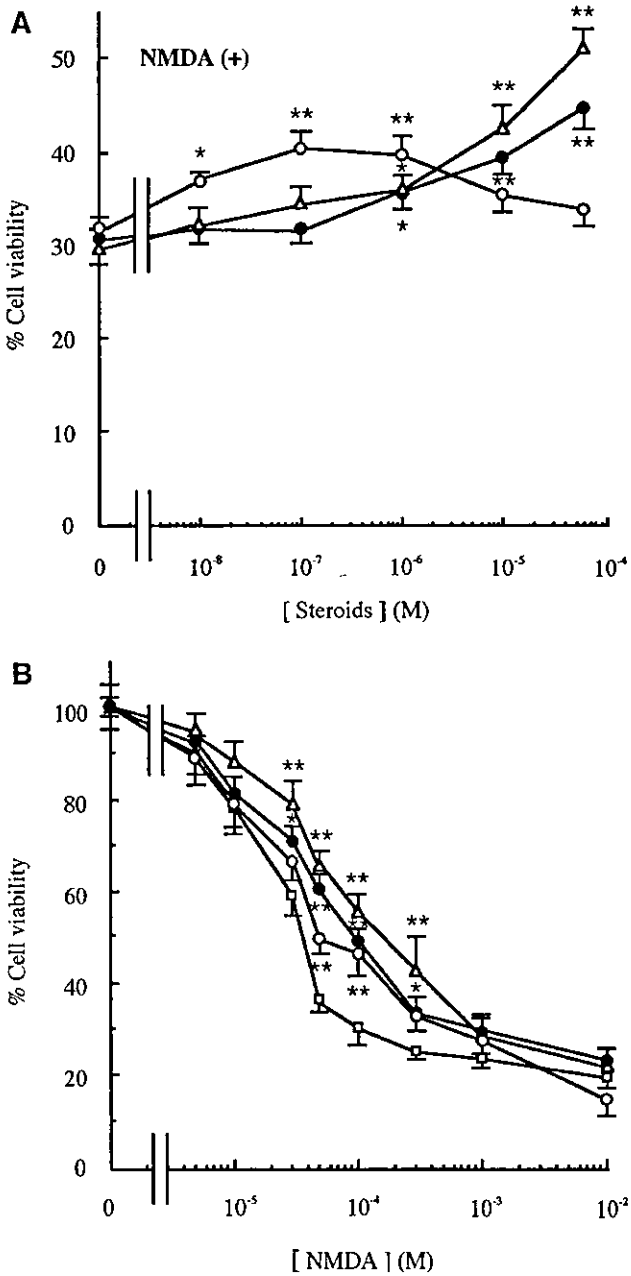

Fig. 1. Neuroprotective effects of β-estradiol, DHEA, and DHEA-S on NMDA-induced neurotoxicity in primary cultured rat hippocampal neurons. A, dose-response curves of steroids. Cells were exposed to 100 μM NMDA for 15 min. β-Estradiol (△), DHEA (●), and DHEA-S (○) were added together with or after NMDA exposure, and effects were assessed 24 h later ($n = 4$). *, $p < 0.05$ and **, $p < 0.01$ compared with the values elicited by 100 μM NMDA without steroids. B, dose-response curves of NMDA. Cells were exposed to NMDA for 15 min. β-Estradiol (△; 60 μM), DHEA (●; 60 μM), DHEA-S (○; 100 nM), and vehicle (□) were added together with or after a 15-min exposure to NMDA, and effects were measured 24 h later ($n = 4$). *, $p < 0.05$ and **, $p < 0.01$ compared with the values elicited by the same concentration of NMDA with vehicle.

β-estradiol and DHEA and 100 nM DHEA-S. These concentrations of steroids were used in subsequent experiments (Figs. 1B, 2B, 4, and 5A; Table 1).

β-estradiol (60 μM), 60 μM DHEA, or 100 nM DHEA-S were added at the same time as or after exposure of cultured neurons to NMDA (shown as steroid treatment 15 min + 24 h; co- and post-treatment in Fig. 2A, 5). Results showed that exposure of cell cultures to NMDA resulted in neurotoxicity in a concentration-dependent manner (Fig. 1B; □). All three steroids shifted the concentration-response curves for NMDA neurotoxicity to the right (Fig. 1B). β-Estradiol and DHEA exerted significant neuroprotective effects against neurotoxicity induced by 30 to 300 μM NMDA. DHEA-S also exerted a neuroprotective effect on neurotoxicity induced by 60 to 100 μM NMDA. In all subsequent experiments in Figs. 1B, 2B, and 3C, and Table 1, we used 100 μM NMDA to assess the neuroprotective effects of steroids.

Because it was not clear whether the neuroprotective effects were related to short- or long-term actions of steroids, we examined kinetics of steroid treatment. The time course is shown in Fig. 2A. β-estradiol (60 μM), 60 μM DHEA, and 100 nM DHEA-S were added either 24 h before NMDA exposure (pretreatment in Fig. 2A, 2), at the same time as NMDA exposure (cotreatment in Fig. 2A, 3), or after NMDA exposure (post-treatment in Fig. 2A, 4) or applied during and after NMDA exposure (co- and post-treatment in Fig. 2A, 5). Time-course studies revealed that only cotreatment with any of the three steroids together with NMDA exposure, but not pre- or post-treatment, induced significant neuroprotective effects, as shown in Fig. 2B. These results indicate that the steroid treatment schedule is important for the neuroprotective effects against NMDA-induced neurotoxicity.

Effects of β-Estradiol, DHEA, and DHEA-S on the NMDA-Induced Increases in $[Ca^{2+}]_i$. It has been proposed that NMDA-induced neurotoxicity is caused by increases of $[Ca^{2+}]_i$ and prevented by direct inhibition of NMDA receptors (Lei et al., 1992; Weaver et al., 1997). We therefore investigated the effects of β-estradiol, DHEA, and DHEA-S on the NMDA-induced increase in $[Ca^{2+}]_i$. The mean increase in $[Ca^{2+}]_i$ induced by 10 μM NMDA for 30 s was 80.3 ± 2.9 nM, and more than 80% of the monitored cells were responsive. In Fig. 3, we routinely used a response to 10 μM NMDA, and the response was fully recovered within 10 min of the application of NMDA. Therefore, 15 min after measuring the first response to NMDA, the cells were stimulated again for 30 s in the presence of β-estradiol, DHEA, and DHEA-S to examine their effects on the NMDA response. Cells were perfused with steroids for 5 min before the second application of NMDA. A typical example is shown in Fig. 3A. To exclude the influence of DMSO, cells were also exposed to BSS with 0.5% DMSO for 5 min before each stimulation with NMDA in the steroid-related experiments. The effects of steroids were calculated by comparing differences between the first and the second responses.

As shown in Fig. 3B, concentrations greater than 1 μM β-estradiol inhibited the NMDA-induced $[Ca^{2+}]_i$ increases significantly in a concentration-dependent manner, with an IC_{50} value of 53.2 μM. Only high concentrations of DHEA (60 μM) and DHEA-S (30–60 μM) had significant inhibitory effects on the NMDA-induced $[Ca^{2+}]_i$ increases. The inhibitory effects of DHEA and DHEA-S were significantly less than those of β-estradiol (two-way ANOVA analysis: DHEA, $p < 0.01$; DHEA-S, $p < 0.01$). At concentrations of 1 to 60 μM DHEA and 10 to 60 μM DHEA-S, respectively, the inhibitory effects of DHEA and DHEA-S were significantly less potent than those of β-estradiol on NMDA-induced $[Ca^{2+}]_i$ increases (Student's t test). Furthermore, there was a strong correla-

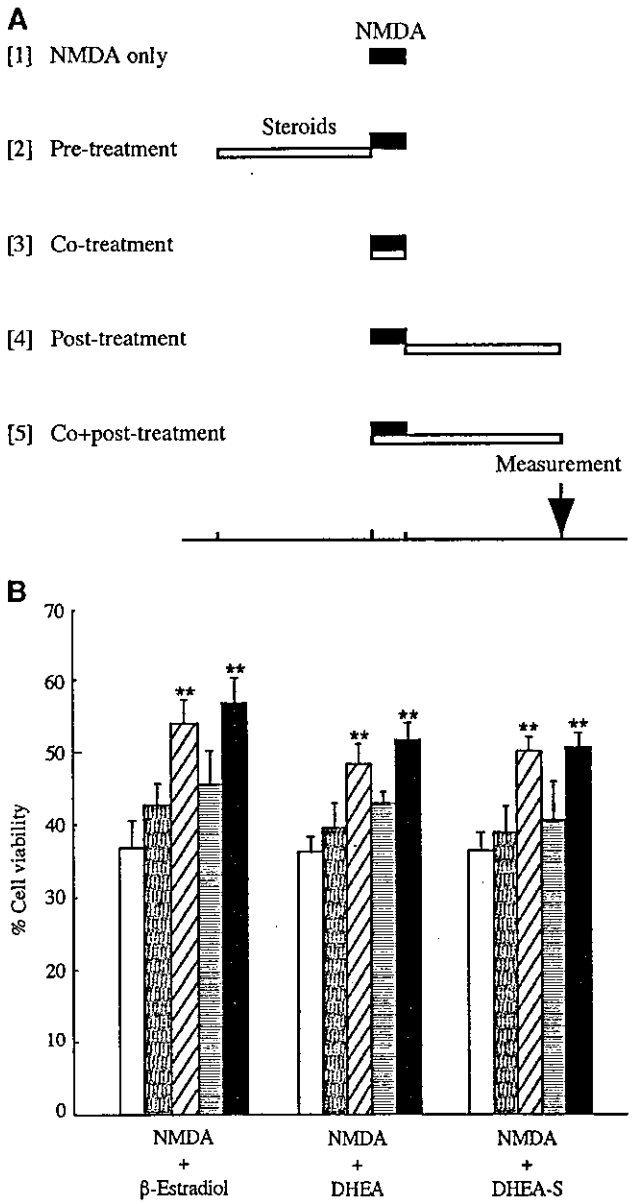

Fig. 2. Short-term effects of β-estradiol, DHEA, and DHEA-S on neuroprotection. A, experimental protocols for treatment with NMDA and steroids. 1) NMDA only; cells were exposed to 100 μM NMDA for 15 min. After 24 h, cell viability was determined by a modified MTT assay. 2) Pretreatment; steroids were added 24 h before NMDA exposure. 3) Co-treatment; steroids were added only during NMDA exposure. 4) Post-treatment; steroids were added for 24 h after NMDA exposure. 5) Co- + post-treatment; steroids were added during and for additional 24 h after NMDA exposure. B, comparisons of neuroprotective effects with different time courses of steroid treatments. Cells were exposed to 100 μM NMDA for 15 min and treated for different times with vehicle, 60 μM β-estradiol, 60 μM DHEA, and 100 nM DHEA-S. NMDA only, □; pretreatment, ▨; cotreatment, ▩; post-treatment, ▤; Co- + post-treatment, ■. Each value represents the mean of values from four independent experiments. **, $p < 0.01$ compared with each NMDA only.

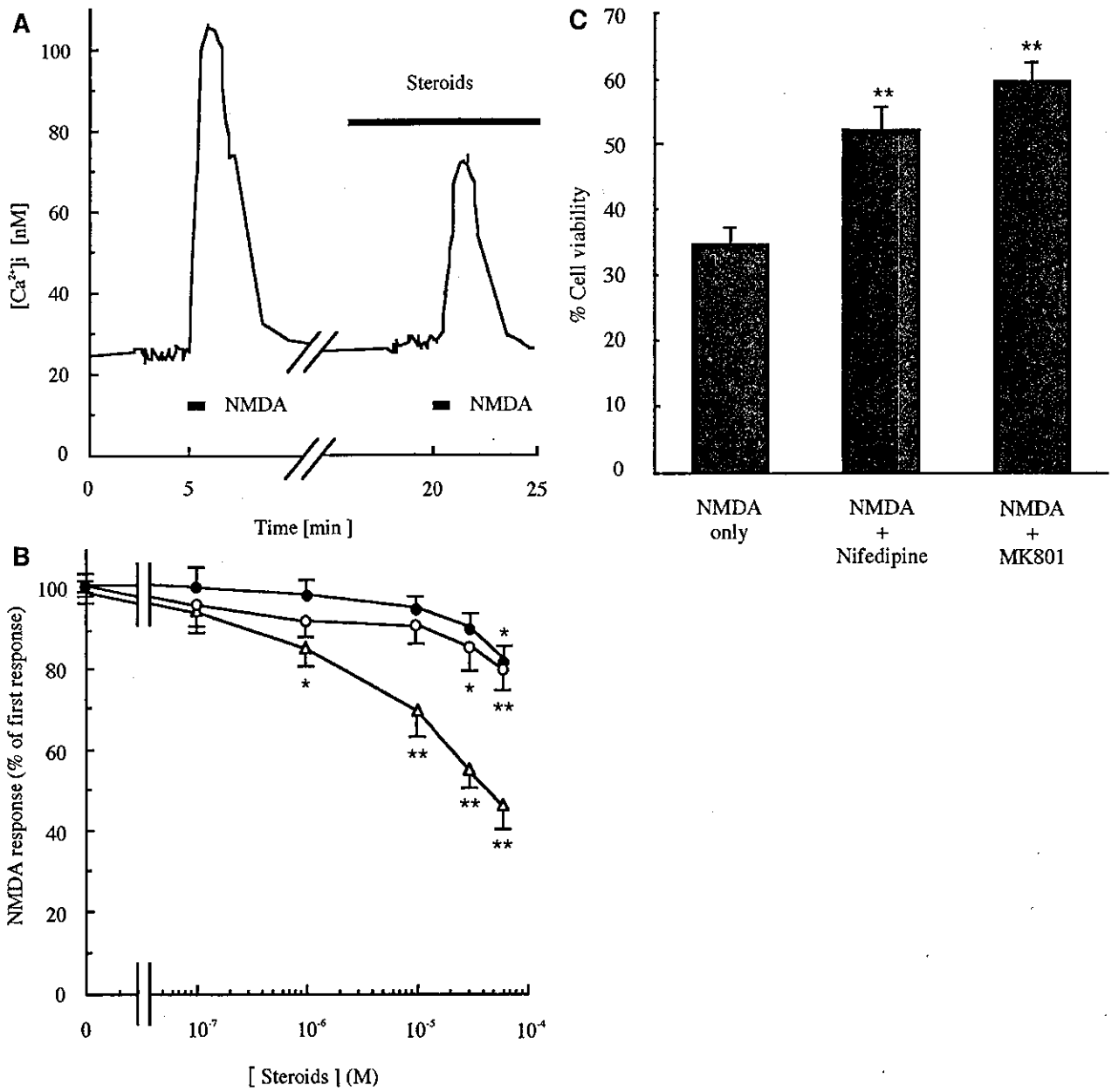

Fig. 3. Neuroprotective effect of β-estradiol through inhibition of NMDA-induced $[Ca^{2+}]_i$ increase and L-type VGCCs. A, typical example of Ca^{2+} response to 10 μM NMDA exposure for 30 s. Cells were perfused with steroids 5 min before the second application of NMDA. Bars represent periods of steroid exposure. B, acute inhibitory effects of β-estradiol (△), DHEA (●), and DHEA-S (○) on 10 μM NMDA-induced $[Ca^{2+}]_i$ increases ($n = 4$). *, $p < 0.05$ and **, $p < 0.01$ compared with the values elicited by 10 μM NMDA stimulation without steroid. C, neuroprotective effects of nifedipine and MK-801 on NMDA-induced neurotoxicity. Nifedipine (10 μM) and 10 μM MK-801 were added at the same time as or after exposure to 100 μM NMDA ($n = 4$). **, $p < 0.01$ compared with NMDA only.

tion between the neuroprotective effects of β-estradiol (Fig. 1A) and inhibitory effects on NMDA-induced $[Ca^{2+}]_i$ increases (Fig. 3B) (Pearson's correlation coefficient: β-estradiol, −0.953, $p = 0.003$) These data suggest that β-estradiol may exert its neuroprotective effect by inhibiting NMDA-induced $[Ca^{2+}]_i$ increases.

Neuroprotective Effects of Nifedipine and MK-801 on NMDA-Induced Neurotoxicity. We previously reported that β-estradiol acutely inhibited L-type voltage-gated calcium channels (VGCCs)-induced Ca^{2+} responses (Kurata et al., 2001). In addition, we have reported that almost half of the NMDA-induced $[Ca^{2+}]_i$ increase was mediated by L-type VGCCs (Kurata et al., 2001). Hence, we showed that β-estradiol exerted neuroprotective effects against NMDA-induced neurotoxicity by inhibiting NMDA-induced Ca^{2+} responses. Therefore, we hypothesized that the neuroprotective effect of β-estradiol was mediated via L-type VGCCs. To examine this possibility, we tested the effect of nifedipine, an L-type VGCCs antagonist, on NMDA-induced neurotoxicity. As shown in Fig. 3C, 10 μM nifedipine as well as 10 μM MK-801,

an NMDA-R antagonist, mediated significant neuroprotective effects against NMDA exposure. These data indicate that β-estradiol may exert neuroprotective effects by inhibiting Ca^{2+} responses via L-type VGCCs.

DHEA Inhibits NMDA-Induced NO Production. To further explore the effects of β-estradiol, DHEA, and DHEA-S on NMDA-induced Ca^{2+}/NO signaling pathways, we next investigated effects of steroids on NMDA-induced NO production. The kinetics of NMDA-induced NO production in the absence of steroids for up to 60 min is shown in Fig. 4A (□). Whereas cotreatment with 100 nM DHEA-S did not significantly affect NMDA-induced NO production at 60 min, cotreatment with 10 μM β-estradiol or 10 μM DHEA resulted in significant inhibition of NO production at 5, 15, 30, and 60 min. In some experiments (Fig. 4 and 5A), a lower concentration of DHEA (10 μM) was used to avoid the inhibitory effect on Ca^{2+} responses occurring at high concentrations (60 μM). This study demonstrated that β-estradiol and DHEA, but not DHEA-S, significantly inhibited NMDA-induced NO production.

Involvement of NOS Activity in the Neuroprotective Effect of DHEA against NMDA-Induced Neurotoxicity. In this study, we showed that DHEA inhibited NMDA-induced NO production (Fig. 4). To confirm that DHEA induced neuroprotection via NO inhibition, we examined the effect of steroids on NOS activity caused by NMDA stimulation. It is well known that the three isoforms of NOS, neuronal NOS (nNOS), inducible NOS, and endothelial NOS (eNOS), are expressed in the CNS. Here, we measured the total NOS activity. Each of the steroids, a nonspecific NOS inhibitor, L-NMMA, and an intracellular Ca^{2+} chelator, BAPTA, were added during a 15-min exposure to 100 μM NMDA, after which NOS activity was determined. As shown in Fig. 5A,

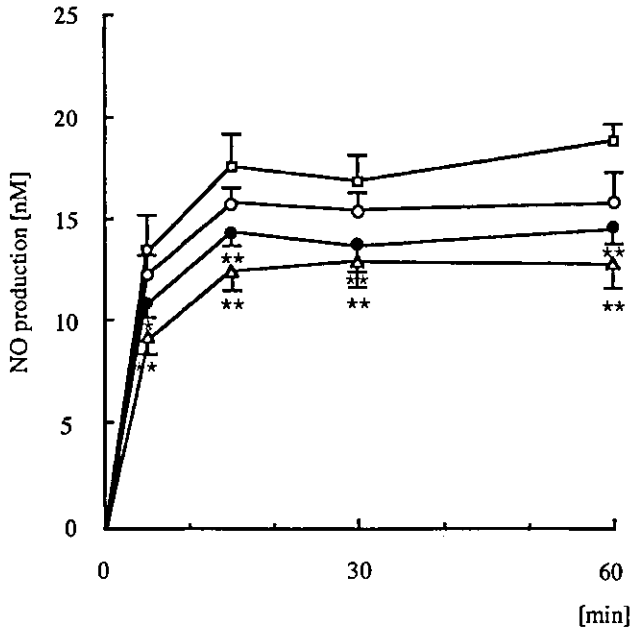

Fig. 4. Inhibition of NMDA-induced NO production by DHEA. Time courses of effects of β-estradiol, DHEA, and DHEA-S on NMDA-induced NO production. Cells were exposed to 100 μM NMDA for 5, 15, 30, or 60 min without steroid (vehicle, □) and in the presence of 10 μM β-estradiol (△), 10 μM DHEA (●), or 100 nM DHEA-S (○), $n = 4$. *, $p < 0.05$ and **, $p < 0.01$ compared with each vehicle at the same time point.

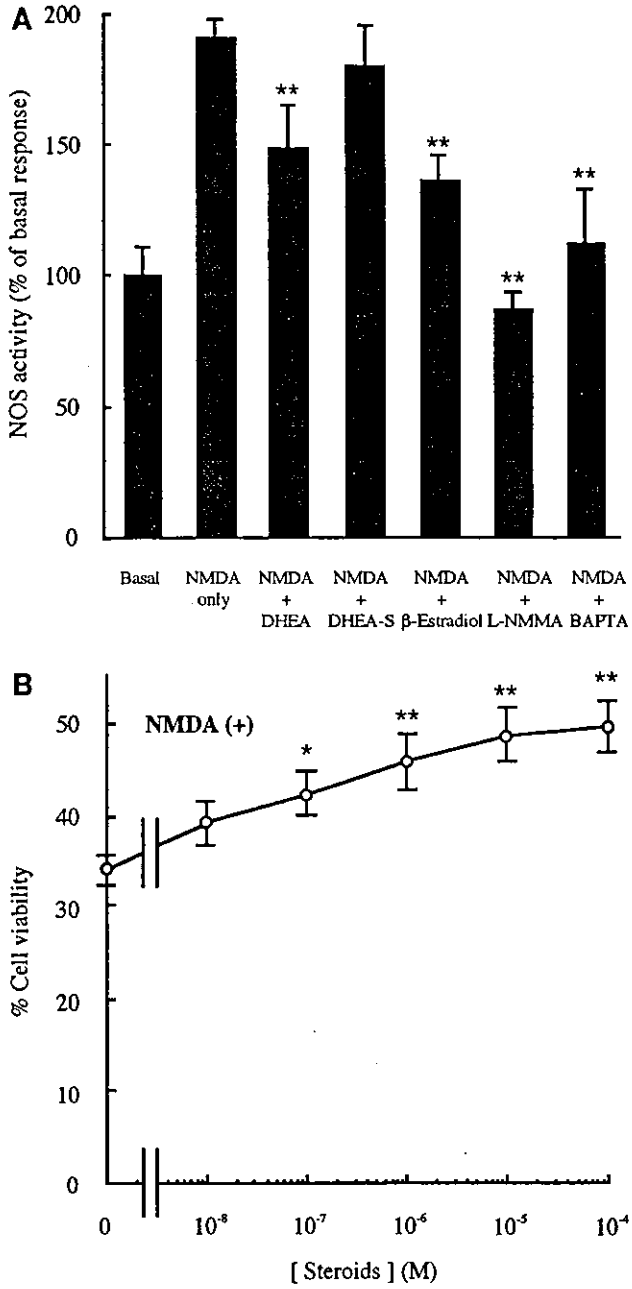

Fig. 5. Neuroprotective effect of DHEA against NMDA-induced neurotoxicity via inhibition of NOS activity. A, effects of β-estradiol, DHEA, DHEA-S, L-NMMA, and BAPTA on NMDA-induced NOS activity. Cells were exposed to 100 μM NMDA for 15 min together with 10 μM DHEA, 100 nM DHEA-S, 10 μM β-estradiol, 10 μM L-NMMA, or 100 μM BAPTA ($n = 4$). **, $p < 0.01$ compared with NMDA only. Basal, basal level of NOS activity (no exposure to NMDA). B, dose-response curves of L-NMMA on NMDA-induced neurotoxicity. Cells were exposed to 100 μM NMDA for 15 min. L-NMMA was added at the same time as or after exposure to 100 μM NMDA ($n = 4$). *, $p < 0.05$ and **, $p < 0.01$ compared with the values elicited by 100 μM NMDA without steroids.

DHEA and β-estradiol, but not DHEA-S, inhibited NMDA-induced NOS activity. NMDA exposure significantly increased NOS activity by approximately 90%, and this was completely blocked by cotreatment with L-NMMA and BAPTA. This indicates that NMDA-induced NOS activity is

Ca^{2+}-sensitive and suggests that the inhibition of NO and NOS by β-estradiol is secondary to its inhibition of the NMDA-mediated Ca^{2+} response. Furthermore, L-NMMA also exerted neuroprotective effects against NMDA-induced neurotoxicity in a concentration-dependent manner in Fig. 5B.

Effects of β-Estradiol, DHEA, and DHEA-S on SNP-Induced Neurotoxicity. Next, we examined whether β-estradiol, DHEA, and DHEA-S affected downstream events in the NMDA-induced Ca^{2+}/NO signaling pathway. SNP is well known as an NO generator that induces cell death through production of NO. Exposure to 1 mM SNP for 15 min resulted in almost the same degree of neurotoxicity as 100 μM NMDA (cell viability 30–40%). β-Estradiol, DHEA, and DHEA-S were added at the same time as or after exposure to 1 mM SNP for 15 min before assay. As shown in Fig. 6, DHEA-S did not exert any protective effects in these experiments. Only 60 μM β-estradiol and DHEA showed neuroprotective effects against SNP-induced neurotoxicity.

Effects of Sig-1R and GABA-A-R Ligands on the Neuroprotective Effects of DHEA-S. We did not observe any action of DHEA-S on the NMDA-induced Ca^{2+}/NO signaling pathway and its downstream events. Therefore, we examined the possibility that the neuroprotective effect of DHEA-S involved its interactions with Sig-1R or GABA-A-R. In the present study, we used two types of σ receptor antagonists, rimcazole and BD1063, and a specific GABA-A-R agonist, muscimol. Rimcazole has high affinities both of Sig-1R and σ-2 receptor (Sig-2R), although it has affinity for dopamine transporter (Gilmore et al., 2004). BD1063, which has preferential affinities for Sig-1R than Sig-2R, is more specific for Sig-1R than rimcazole (Matsumoto et al., 1995). β-Estradiol, DHEA, and DHEA-S were added together with each of 1 μM rimcazole, 1 μM BD1063, and 10 μM muscimol to test their effects on a 15-min exposure to 100 μM NMDA. As shown in Table 1, 1 μM rimcazole, 1 μM BD1063, and 10 μM muscimol treatment did not affect cell viability. However, treatment with rimcazole and BD1063, but not muscimol, partly, but significantly, reversed the neuroprotective effect of DHEA-S against the NMDA-induced neurotoxicity. On the other hand, the neuroprotective effects of DHEA or β-estradiol were not affected by rimcazole, BD1063, or muscimol. These results suggest that DHEA-S may exert its neuroprotective effects against NMDA-induced neurotoxicity partly via Sig-1R, but not GABA-A-R.

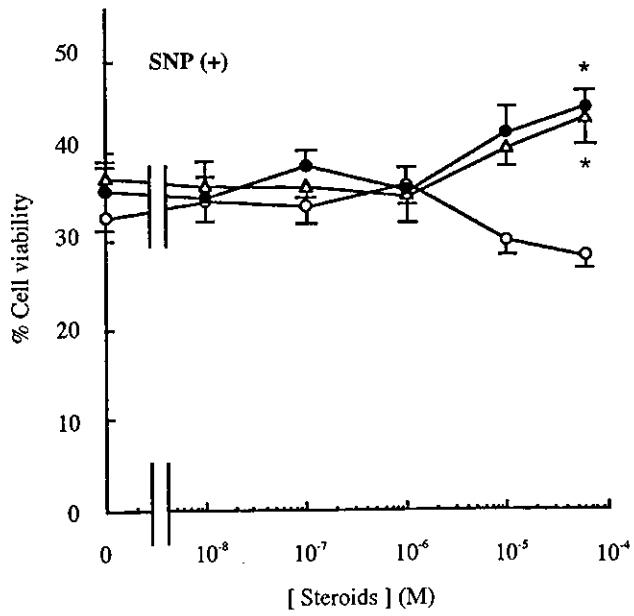

Fig. 6. Effects of β-estradiol, DHEA, and DHEA-S on SNP-induced neurotoxicity. Cells were exposed to 1 mM SNP for 15 min. Twenty-four hours later, cell viabilities were determined by the modified MTT assay. β-Estradiol (△), DHEA (●), and DHEA-S (○) were added at the same time as or after exposure to SNP ($n = 4$). *, $p < 0.05$ compared with the values elicited by 1 mM SNP exposure without steroids.

Discussion

We have demonstrated that β-estradiol, DHEA, and DHEA-S exert neuroprotective effects against NMDA-induced neurotoxicity in hippocampal neurons. These neuroprotective effects of all three steroids were exerted by treatment for short periods of time, although they were mediated by different mechanisms, including the Ca^{2+}/NO signaling pathway and the Sig-1R.

The present results show that the neuroprotective effect of β-estradiol against NMDA-induced neurotoxicity is closely correlated with its inhibitory effects on NMDA-induced $[Ca^{2+}]_i$ increases, indicating that β-estradiol exerts its neuroprotective effect mainly by inhibiting NMDA-induced $[Ca^{2+}]_i$ increases. We previously reported that almost half of the NMDA-induced $[Ca^{2+}]_i$ increase was mediated by L-type VGCCs in hippocampal neurons (Kurata et al., 2001). Therefore, it is hypothesized that β-estradiol acts at the level of NMDA-R or L-type VGCCs to reduce Ca^{2+} influx, thereby exerting a neuroprotective effect that is independent of estrogen receptors. A previous report also suggested that neuroprotective effects of β-estradiol against NMDA-induced neurotoxicity did not require any participation by nuclear estrogen receptors, because estrogen receptor antagonists or estrogen analogs did not interfere with such protective effects (Weaver et al., 1997; Xia et al., 2002). Furthermore, neither estrogen receptor antagonists nor protein synthesis inhibitors block neuroprotection by β-estradiol against glutamate neurotoxicity in mesencephalic neurons (Sawada et al., 1998). In addition, a number of studies suggested that estradiol had nongenomic actions modulating membrane receptor functions, including NMDA-R and VGCCs, within milliseconds to minutes (Moss et al., 1997), suggesting that nongenomic actions of β-estradiol are among the most important neuroprotective mechanisms against NMDA-induced neurotoxicity. As for VGCCs, β-estradiol reduced Ba^{2+} currents of L-type VGCCs (Mermelstein et al., 1996). Our results showed that an L-type VGCCs antagonist (nifedipine) exerted neuroprotective effects on NMDA-induced neurotoxicity. Furthermore, it is reported that L-type VGCCs antagonists protect neurons from ischemic damage (Campbell et al., 1997). Thus, our results suggested that β-estradiol may inhibit L-type VGCCs-mediated Ca^{2+} influx, which results in a neuroprotective effect against NMDA-induced neurotoxicity. On the other hand, although there are few studies that refer to direct modulatory effects of β-estradiol on NMDA-R, Weaver et al. (1997) reported that β-estradiol protected against NMDA-induced excitotoxicity by direct inhibition of NMDA-R, suggesting a possible direct interaction between

β-estradiol and NMDA-R. Further studies are required to clarify the precise mechanisms of β-estradiol actions on L-type VGCCs and NMDA-R.

β-Estradiol has been reported to prevent neuronal death induced by various neurotoxins such as glutamate, oxidative stress, or β-amyloid (Singer et al., 1996; Behl et al., 1997; Gridley et al., 1998). In previous studies, low concentrations of β-estradiol (15–50 nM) were added before or at the same time as the neurotoxin and protective effects assessed over 20 to 72 h. Here, neuroprotective effects were mediated through mechanisms such as the up-regulation of Bcl-2 expression (Dubal et al., 1999). In our experiments, addition of low concentrations of β-estradiol (10–100 nM) together with or after the insult, failed to act as a neuroprotectant (Fig. 1A). Pretreatment with a high concentration of β-estradiol (60 μM) also failed to reveal any neuroprotective effects (Fig. 2B). We did not examine effects of pretreatment with low concentrations of β-estradiol in this study. The results presented here suggest that the neuroprotective mechanisms operating with pretreatment may differ depending on the concentrations of β-estradiol. It is important to investigate the effects of β-estradiol at higher concentrations for the reasons that follow (Kurata et al., 2001). 1) Local concentrations of estradiol could be markedly increased because of the high levels of expression of estrogen-synthesizing enzymes in specific areas, including the hippocampus (MacLusky et al., 1994). 2) The actual concentration of free hormone may be many times greater than the plasma concentration, because protein-bound hormone can function in vivo as a free fraction (White et al., 1995).

DHEA (1–60 μM) showed significant neuroprotective effects against NMDA-induced neurotoxicity, whereas 1 to 30 μM DHEA did not inhibit the NMDA-induced $[Ca^{2+}]_i$ increases. We also demonstrated that 10 μM DHEA inhibited NMDA-induced NOS activity and NO production. Furthermore, L-NMMA, an NOS inhibitor, exerted a significant neuroprotective against NMDA-induced neurotoxicity (Fig. 5B), suggesting a possible involvement of NOS activity on the neuroprotection by DHEA against NMDA-induced neurotoxicity. It is well known that two isoforms of NOS, nNOS and eNOS, are regulated by Ca^{2+} and expressed constitutively in neurons. In the CNS, nNOS is exclusively localized to discrete populations of neurons in the cortex, basal forebrain, brain stem, and hippocampus (Bredt et al., 1991). A number of studies have demonstrated that nNOS gene knock-out confers resistance to cerebral ischemia (Huang et al., 1994) and glutamate neurotoxicity (Ayata et al., 1997), supporting the possibility that of the three types of NOS, it is nNOS that plays the key role in DHEA-mediated neuroprotection. Because previous reports showed that DHEA inhibited lipopolysaccharide-induced microglial NO production by inhibiting inducible NOS (Wang et al., 2001) and that DHEA activated eNOS through a specific plasma membrane receptor coupled to $G_{\alpha i2,3}$ (Liu and Dillon, 2002) or through mitogen-activated protein kinases (Simoncini et al., 2003), the modulatory effect of DHEA on the NOS activity remains undetermined. Furthermore, whether the inhibitory effect on NOS activity by L-NMMA is the same mechanism as that produced by DHEA basically needs to be addressed.

β-Estradiol and DHEA at the concentration of 60 μM also showed significant neuroprotective effects against SNP-induced neurotoxicity (Fig. 6), suggesting that β-estradiol and DHEA have the ability to scavenge free radicals at micromolar concentrations. It is well known that the NMDA-induced neurotoxicity involves various systems, including not only $[Ca^{2+}]_i$ homeostasis and NO generation but also ATP production and generation/detoxification of reactive oxygen species. The mitochondrial function is most important to prevent the NMDA-induced neurotoxicity, because the mitochondria play a key role on production of reactive oxygen species (Lee et al., 2002). Furthermore, as reported previously, DHEA and estradiol could partly preserve the brain mitochondrial functions altered by ischemic damages with a concentration manner (Morin et al., 2002). Therefore, in future studies, it is important to use fractions of mitochondria to avoid nonspecific effects of steroids especially at high concentrations, such as direct actions on many membrane receptors (Rupprecht and Holsboer, 1999) and NADH dehydrogenase (Sonka et al., 1978).

The σ receptor ligands prevent neuronal death associated with glutamate cytotoxicity. There is increasing evidence suggesting that the neuroprotective effects are mediated via Sig-1R (DeCoster et al., 1995). DHEA-S acts as a Sig-1R agonist and exerts facilitatory actions on NMDA-mediated glutamatergic and noradrenergic neurotransmission (Monnet et al., 1995; Maurice and Lockhart, 1997). Because DHEA-S has a high affinity for Sig-1R (Monnet et al., 1995), our results suggest that its neuroprotective effect may be mediated via Sig-1R, at least in part. Recent studies have shown that intracellular Sig-1R located at the endoplasmic reticulum regulate several components, such as Ca^{2+}, phospholipase C, and protein kinase C (PKC) activity (Morin-Surun et al., 1999). Furthermore, an elevation of PKC activity could increase the neurotoxicity mediated by NMDA-R activation (Wagey et al., 2001). The regulation of dopamine release by Sig-1R ligands is dependent on PKC (Nuwayhid and Werling, 2003). Thus, Sig-1R ligands could regulate PKC activity, resulting in modulation of NMDA-induced neurotoxicity. Therefore, one possibility to explore is that DHEA-S may exert neuroprotective effects against NMDA-induced neurotoxicity by regulating PKC activity via intracellular Sig-1R.

In summary, our results document different neuroprotective mechanisms of β-estradiol, DHEA, and DHEA-S against NMDA-induced neurotoxicity. All three steroids possess neuroprotective properties as short-term actions. β-Estradiol, DHEA, and DHEA-S may, respectively, exert their neuroprotective effects by reducing Ca^{2+} signaling via NMDA-R and/or L-type VGCCs, inhibiting NOS activity, and mediating partly by Sig-1R. Our findings may provide some important insights into the therapeutic mechanisms of neurosteroids in stress disorders.

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Lithium and protein phosphatases: apoptosis or neurogenesis?

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Abstract

Whereas the effects of lithium on the expression and activity of kinases are extensively examined, relatively little is known about the effect of lithium on the expression and activity of protein phosphatases (PPs). It has been demonstrated that PPs dephosphorylate a number of transcription factors and kinases, leading to decreased gene transcription in the rat brain. Since long-term administration of lithium is required to obtain a therapeutic and prophylactic effect, it is conceivable that regulation of neural plasticity mediated by changes in gene transcription plays an important role in its therapeutic action. In this context, examining the effect of lithium on the expression and activity of PPs may promote our understanding of the molecular action of lithium. In this review, we summarize the results of our studies examining the influence of lithium on the expression and the serine/threonin phosphatase activity of PP2A and 2B, and CREB phosphorylation in the rat brain. We also discuss the putative role of the increased PP2A activity in the therapeutic action of lithium.

Keywords: Apoptosis; Calcineurin; cAMP response element binding protein (CREB); Lithium; Neurogenesis; Protein phosphatase

1. Introduction

An accumulation of evidences derived from clinical studies of mood disorders indicates that long-term administration of mood stabilizers is required to alleviate both manic and depressive features [1,2]. Based on this evidence, it has been postulated that changes in neuronal gene expression induced by repeated, treatments with mood stabilizers, such as lithium or valproate, are involved in the action of these drugs. In this context, there have been numerous preclinical studies examining the effect of chronic administration of mood stabilizers on gene expression [3–7]. For example, Chen and his coworkers [8] demonstrated that long-term treatment with lithium or valproate increased the expression of the antiapoptotic gene, bcl-2, in the rat frontal cortex through an increase in the DNA-binding activity of the transcription factor, polyomavirus enhancer-binding protein 2β. In addition, chronic treatment with these agents led to increased expression of the neuroprotective genes, brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in the rat brain [9,10]. In contrast, expression of the proapoptotic gene, Bax, is decreased in response to long-term administration of lithium [11]. Taken together, the results suggest that several genes involved in neuroprotection are modulated by lithium or valproate, and that the effects upon these genes may be involved in the therapeutic action of these mood stabilizers.

The phosphorylation of transcription factors, such as cAMP response element binding protein (CREB), has also been suggested to play an important role in the therapeutic actions of mood stabilizers [5]. In fact, recent studies have revealed that lithium and valproate regulate the activity of various kinases in the rat brain [6]. For instance, both lithium and valproate have been reported to inhibit the activity of glycogen synthase kinase 3 (GSK-3), and subsequently upregulate gene expression mediated the activation of β-catenin in the rat brain [3,12,13]. Furthermore, lithium and valproate increased the levels of active, phosphorylated extracellular signal-regulated kinase (ERK) 44/42 and active, phosphorylated ribosomal protein S6 kinase-1 in the rat hippocampus and frontal cortex [14]. Yuan and his colleagues [15] demonstrated that long-term administration of lithium increases the levels of phosphorylated c-Jun N-terminal kinases (JNKs) significantly and activates JNK signal transduction. Based on this evidence, it

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is conceivable that lithium and valproate regulate gene expression via the activation of several kinases, and that this activity might be associated with the therapeutic action of these mood stabilizers.

In contrast, it has recently been revealed that protein phosphatases dephosphorylate a number of transcription factors and kinases, leading to decreased gene transcription [16,17]. In this context, it is likely that the balance between phosphorylation and dephosphorylation of these factors in the brain regulates neuronal functions via changes in neuronal gene expression. Whereas the influence of mood stabilizers on the activity of protein kinases has been documented [6], relatively little is known of the effect of mood stabilizers on the expression and activity of protein phosphatases in the rat brain [18–20]. The purpose of this review is to summarize the results of our studies examining the influence of mood stabilizers on the expression and the serine/threonin phosphatase activity of protein phosphatase 2A, 2B (calcineurin), and the expression and phosphorylation state of CREB in the rat brain. Furthermore, we discuss the possible role of mood stabilizers in neuroprotection.

2. Protein phosphatase 2A and lithium

Protein phosphatase 2A (PP2A) has been conserved from yeast to mammals. Protein phosphatase 2A is found as a heterodimer, containing a catalytic subunit C and regulatory subunit A, or as a heterotrimer of subunits A, C, and B. The B subunit has been shown to affect the activity and substrate selectivity of PP2A [16,17,21]. Recent studies have revealed that PP2A plays pivotal roles in the regulation of cell growth, gene expression, and development [17]. For example, the dephosphorylation of CaMK II by PP2A has been reported to influence synaptic strength, as determined by the measurement of long-term depression (LTD) [22]. It has further been suggested that PP2A might regulate gene transcription mediated by the signaling complex of CaMK IV and CREB or of p70 S6 kinase [23,24]. In this context, it is postulated that PP2A may play a fundamental role in gene induction in response to extracellular stimuli.

Recently, the influence of acute and chronic administration of lithium on the expression and the serine/threonin phosphatase activity of PP2A in the rat brain has been extensively examined in our laboratory. To examine the levels of PP2A expression, we performed western blot and immunohistochemical analyses using an anti-goat PP2A C subunit antibody. To examine the level of serine/threonine phosphatase activity of PP2A, the amount of free phosphate derived from the synthetic phosphopeptide RRA(pT)VA (Promega, Madison, WI) was measured by the absorbance of the molybdate-malachite green-phosphate complex using a micro plate reader. Rats were kept on a 0.2% lithium carbonate-containing diet, and normal drinking water and a 1.5% NaCl solution were available to all rats.

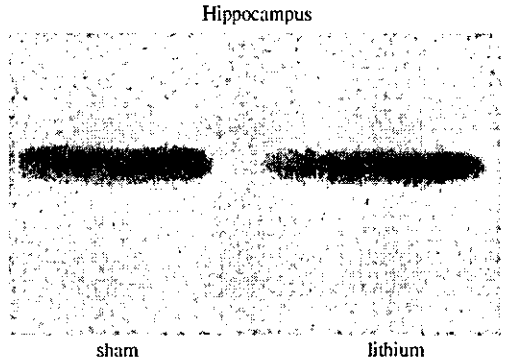

Fig. 1. Western blot analysis of the influence of repeated treatments with lithium (14 d) on PP2A immuoreactivity in the rat hippocampus. Representative immunoblots of the catalytic subunit C of PP2A in the rat hippocampus.

Western blot analyses revealed that the treatment with lithium for either 1 or 14 days had no effect upon the levels of PP2A immunoreactivity in the rat frontal cortex or hippocampus (Fig. 1). Similarly, immunohistochemical analyses demonstrated that administration of lithium for 1 or 14 days had no effect upon PP2A immunoreactivity in either the frontal cortex or hippocampus (Fig. 2).

In contrast, administration of lithium for either 1 or 14 days significantly upregulated the serine/threonine phosphatase activity of PP2A in the rat frontal cortex and hippocampus [20]. Thus, lithium induces the serine/threonine phosphatase activity of PP2A in the rat frontal cortex and hippocampus without affecting its the expression of this enzyme in these regions.

3. Protein phosphatase 2B (calcineurin) and lithium

The Ca^{2+}/calmodulin-dependent protein phosphatase 2B, calcineurin (CaN), is a heterodimer comprising a 61-kDa catalytic subunit (CaN A) and a 19-kDa regulatory subunit (CaN B), that dephosphorylates various substrate proteins [25,26]. The A subunit of CaN is highly expressed in the rat frontal cortex, striatum, and hippocampus [27]. As is the case with PP2A, CaN plays an important role in the synaptic plasticity of hippocampal function, since the sustained phosphorylation of CREB caused by the inhibition of CaN activity promotes the development of LTP [28]. In addition, the dephosphorylation of CREB by the activated inhibitor-1 (I-1) through the dephosphorylation by CaN is, at least in part, involved in the reduction in the rate of gene transcription. In this context, both PP2A and CaN are thought to be involved in the regulation of gene transcription.

Recently, the influence of acute and chronic administration of lithium on the expression and the serine/threonin phosphatase activity of CaN in the rat brain have been extensively examined in our laboratory.

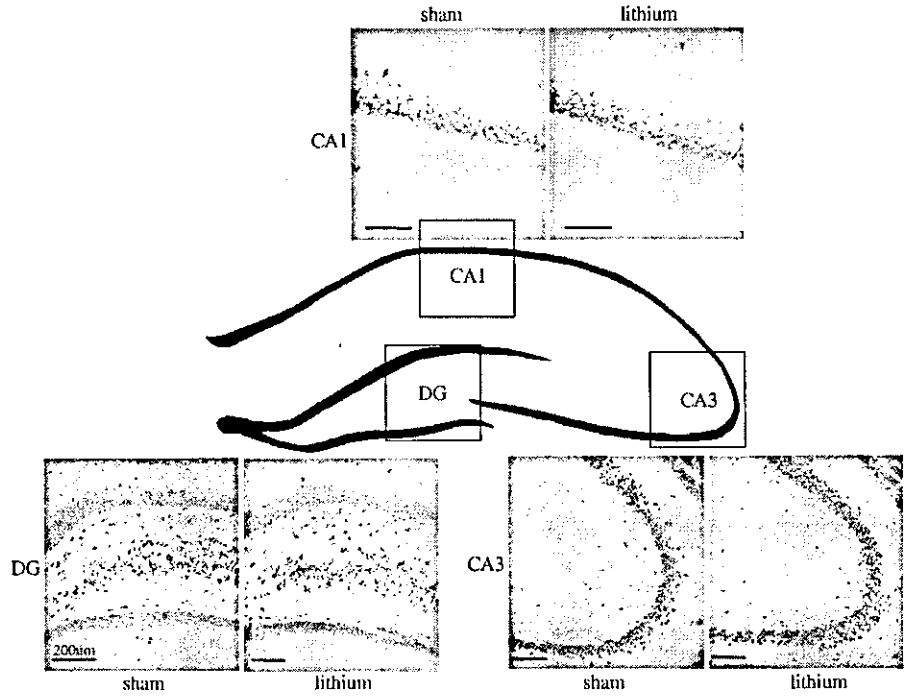

Fig. 2. Immunohistochemical analysis of the influence of repeated treatments with lithium (14 d) on PP2A expression in the rat hippocampus. CA, cornu ammonis; DG, dentate gyrus.

To examine the levels of CaN expression, we performed Northern blot analysis of mRNA expression using a probe from the A subunit of CaN. To examine the level of serine/threonine phosphatase activity of CaN, we used a similar method to that used above for the PP2A study, except that we substituted a different reaction buffer.

Treatment with lithium for either 1 or 14 days had no effect upon the levels of CaN mRNA in the rat frontal cortex and hippocampus (Fig. 3). Similarly, treatment with lithium had no effect upon the serine/threonine phosphatase activity of CaN in these brain regions [18]. Thus, lithium does not affect either the expression or serine/threonine phosphatase activity of CaN in the rat frontal cortex or hippocampus.

4. Protein phosphatases and CREB

It is well known that the phosphorylation of CREB at Ser 133 plays an important role in the transcription of genes, such as c-fos or BDNF, through interaction of the phospho-CREB homodimer with the CRE site located in the promoter region of such genes [29–33]. The activation of several protein kinases, including protein kinase A, calcium/calmodulin-dependent protein kinase II and IV, 90 kDa ribosomal S6 kinase, has been reported to enhance the phosphorylation of CREB [34,35]. On the other hand, various studies have suggested that PPs can downregulate the activity of these protein kinases by their dephosphorylation [16]. For example, the dephosphorylation of I-1 by CaN can increase PP1 activity and subsequently lead to increased dephosphorylation of CREB [36,37]. CaMK II and IV are also dephosphorylated by PP2A through an increase in the intracellular Ca^{2+} concentration, leading to a suppression in the rate of CREB phosphorylation [23,38].

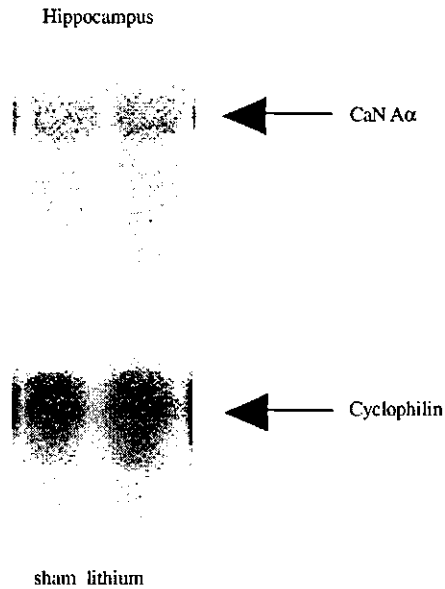

Fig. 3. Northern blot analysis of the influence of repeated treatments with lithium (14 d) on the levels of PP2B mRNA in the rat hippocampus. Representative Northern blot of the subunit A mRNA of PP2B and cyclophilin mRNA in the rat hippocampus.

Various extracellular stimuli, such as neurotrophic factors, result in the activation of the mitogen-activated protein kinase (MAPK)—extracellular signal-regulated kinase (Erk)—90 kDa ribosomal S6 kinase (Rsk) cascade, which also increases the levels of phospho-CREB [5]. In contrast to the phosphorylation of CREB by the MAPK-Erk-Rsk cascade, PP2A has been reported to dephosphorylate protein kinases at several points in this cascade and consequently downregulate the phosphorylation of CREB [16]. In this context, it is conceivable that PPs are involved in the regulation of CRE-mediated gene expression.

5. Lithium and CREB phosphorylation

As described above, the influence of lithium on the phosphorylation state of CREB, which is in turn regulated by the balance between the activity of protein kinases and phosphatases, is thought to be an important component of its mechanism of therapeutic action via its effects upon gene expression. In this context, we performed Western blot analysis to examine whether long-term administration of lithium resulted in changes in the steady-state level of phosphorylated CREB in the rat brain. Western blot analysis revealed a phospho-CREB immunoreactive band of approximately 43 kDa (Fig. 4A). Administration of lithium for 14 days had no effect on the level of phospho-CREB immunoreactivity in the rat frontal cortex or hippocampus

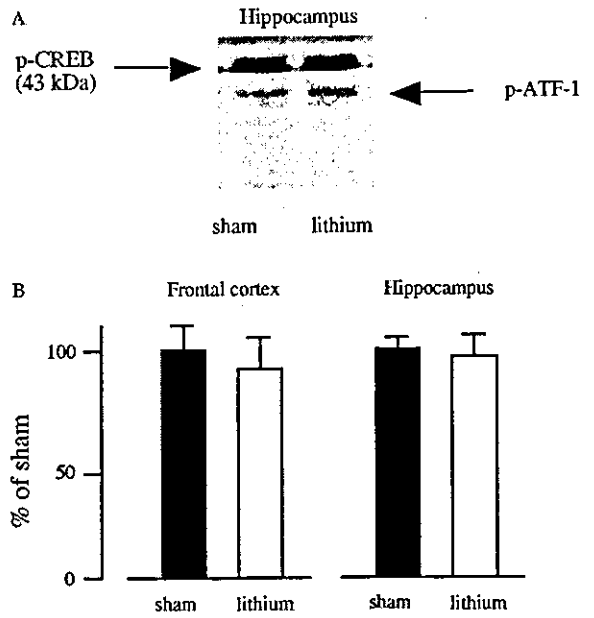

Fig. 4. Western blot analysis of the influence of repeated treatments with lithium (14 d) on the levels of phospho-CREB immunoreactivity in the rat hippocampus. (A) Representative immunoblots of phospho-CREB in the rat hippocampus. (B) Quantification of phospho-CREB immunoreactivity in response to repeated treatments with lithium. Data are presented as percentage of sham-treated rats and are the mean ± SEM of six rats for each treatment group.

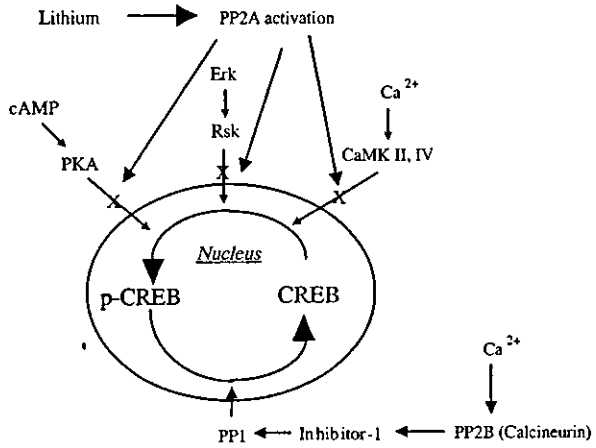

Fig. 5. Regulation of CREB phosphorylation and dephosphorylation by lithium treatment through the balance between the activity of protein kinases and phosphtases. CaM K; calcium/calmodulin-dependent protein kinase, CREB; cAMP response element binding protein, ERK; extracellular signal-regulated kinase, PKA; protein kinase A, PP; protein phosphatase, Rsk; 90 kDa ribosomal S6 kinase.

(Fig. 4B). Thus, repeated treatment with lithium does not affect the phosphorylation of CREB in the rat brain [39].

Although several studies have examined the effect of lithium on the phosphorylation state of CREB, the results are still inconclusive. Chuang and his colleagues demonstrated that chronic lithium treatment enhanced the phosphorylation of CREB in cultures of cerebellar granular neurons and human neuroblastoma SH-SY5Y cells [19,40]. Similary, Grimes and Jope demonstrated that lithium increased the DNA binding activity of CREB in human neuroblastoma SSH-SY5Y cells. Conversely, Young and his coworkers demonstrated that chronic lithium treatment decreased CREB phosphorylation in cultures of human neuroblastoma SH-SY5Y cells and in the rat brain [41,42]. Our results are inconsistent with these previous findings. Since various signal transduction pathways involving numerous protein kinases and phosphatases are involved in the phosphorylation of CREB, it is likely that different experimental procedures may lead to the discordant effects of lithium on CREB phosphorylation. We speculate that the effect of lithium on the activity of kinases may have been counteracted by its effects on the activity of phosphatases under the experimental conditions we used. Thus, no significant change in the steady-state level of phosphorylation of CREB was found in response to long-term lithium administration.

Based on the influence of lithium on the activity of PPs, we summarize the plausible action of lithium on the regulation of CREB phosphorylation (Fig. 5).

6. Lithium and apoptosis

It has been postulated that protein phosphatases play key roles in apoptosis [43,44]. In Fig. 6, we propose mechanisms

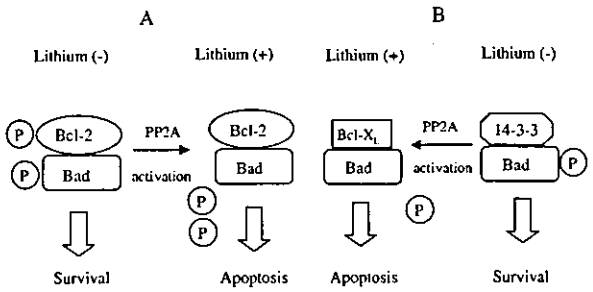

Fig. 6. Regulation of apoptotic signal transductions by lithium treatment through Bcl-2 (A) (modified from Garcia et al.), and Bad (B) (modified from Klumpp and Krieglstein).

by which PP1, PP2A, and PP2B induce apoptosis. Growing evidence suggests that the dephosphorylation of the anti-apoptotic protein, Bcl-2, plays an important role in the induction of apoptosis [45,46]. In particular, Ruvolo et al. [47] demonstrate that the rapid Bcl-2 dephosphorylation in response to ceramide induces apoptosis and that the activation of mitochondrial PP2A, which is colocalized with Bcl-2 at the mitochindrial membrane, is closely involved in this ceramide-induced apoptosis. In addition to Bcl-2, PP1, PP2A, and PP2B are responsible for the dephosphorylation of the proapoptotic protein, BAD [48–50]. It has been reported that phosphorylated BAD located at the mitochondrial surface can bind to cytoplasmic 14-3-3 proteins, which in turn leads to the inhibition of the apoptotic activity of BAD [51]. On the other hand, dephosphorylated BAD, arising from the action of protein phosphatases, can bind to Bcl-XL and subsequently inhibit its antiapoptotic activity [44]. In this context, our finding that lithium administration increases the activity of PP2A in the rat brain suggests that it may result in the enhancement of the apoptotic activity mediated by BAD, due to enhanced dephosphorylation of BAD under these conditions.

The results of our studies indicate that administration of lithium for either 1 or 14 days significantly upregulates the activity of PP2A, but not PP2B [18,22]. These data suggest that lithium has an apoptotic action in the central nervous system. This finding contrasts with previous evidence indicating that lithium has potent neuroprotective effects.

However, it remains plausible that lithium may have an apoptotic effect. It is well known that long-term lithium administration is required to obtain a therapeutic and prophylaxic effect in the treatment of bipolar mood disorder. If long-lasting neuroprotective and neurotprophic effects, such as neurogenesis, are continually induced during lithium treatment, neuronal hyperplasia may result. However, there is no evidence that long-term administration of lithium increases the prevalence of hyperplastic change in the central nervous system, although chronic lithium treatment (4 w) is reported to increase the grey matter volume in patients with bipolar disorder [52]. In contrast, it is well known that lithium treatment induces parathyroid hyperplasia and epidermal hyperplasia [53–58]. In this context, apoptotic effects induced by the activation of PP2A in response to lithium might play an important role in the control of various cellular processes, such as degradation and regeneration.

7. Conclusion

Numerous studies have postulated that potent neurotrophic and neuroprotective effects are an important component of the therapeutic action of chronic lithium treatment. Changes in neuronal gene expression resulting from the activation of various protein kinases may represent one means by which lithium exerts its therapeutic action. However, the regulation of protein phosphorylation by protein kinases and phosphatases plays an important role in the morphogenesis of neurons and glia cells. In this context, it is likely that the activation of protein phosphatases by lithium may regulate the neurogenetic action of lithium. Further studies examining the influence of lithium on apoptosis and neurogenesis in protein phosphatase knock-down animals using double-stranded RNA-mediated RNA interference should help us elucidate the role of protein phosphatases in the therapeutic action of this mood-stabilizing agent.

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