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Regulation of *N*-methyl-D-aspartate cytotoxicity by neuroactive steroids in rat cortical neurons

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Abstract

We investigated the effects of neuroactive steroids on *N*-methyl-D-aspartate (NMDA) cytotoxicity in cultured rat cortical neurons. 3α-Hydroxy-5β-pregnan-20-one sulfate (3α5βS) attenuated, whereas pregnenolone sulfate and pregnenolone hemisuccinate exacerbated, NMDA neurotoxicity in cortical slice cultures. These actions of steroids were not affected by inhibition of protein synthesis, by blockade of $GABA_A$ receptors, or by blockade of σ receptors. In addition, the actions of steroids were not affected by manipulation of cyclic AMP levels or protein kinase C activity. We found that 3α5βS attenuated and pregnenolone hemisuccinate augmented NMDA-induced currents in cortical neurons, whereas pregnenolone sulfate exerted no significant effect. Fluorometric measurements revealed that 3α5βS attenuated and pregnenolone hemisuccinate augmented glutamate-induced increase in intracellular Ca^{2+}. Pregnenolone sulfate slowed the decay of Ca^{2+} increase induced by glutamate, without significant effect on the peak amplitude of Ca^{2+} increase. These results indicate that neuroactive steroids affect NMDA cytotoxicity by modulation of Ca^{2+} influx through NMDA receptor-associated channels.

Keywords: Cerebral cortex; Excitotoxicity; Neurosteroid; NMDA (*N*-methyl-D-aspartate); Organotypic slice culture

1. Introduction

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system, but exposure of neurons to glutamate or other excitatory amino acids in excess may result in cell death through a process termed excitotoxicity (Choi, 1988). An *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors is considered to play a pivotal role in triggering excitotoxicity, which has been implicated in various neurodegenerative disorders including hypoxic–ischemic events, trauma, Alzheimer's disease, Huntington's disease and Parkinson's disease (Meldrum and Garthwaite, 1990). Excessive stimulation of NMDA receptors, as a result of efficient incorporation of Ca^{2+} into neuronal cytoplasm, disrupts intracellular Ca^{2+} homeostasis, leading to the induction of cell death.

Neuroactive steroids refer to steroidal compounds that exert physiological/pharmacological actions on neuronal cells in the central and peripheral nervous system (Gasior et al., 1999). Among them are neurosteroids that are produced within the nervous system by metabolism of cholesterol or downstream precursors derived from peripheral sources (Baulieu, 1997). In general, various actions of classical steroid hormones are exerted through the mechanisms that involve their binding to intracellular receptors, translocation of receptors to the nucleus and initiation of de novo protein synthesis. Although several lines of evidence suggest that neuroactive steroids can interact with intracellular steroid receptors (Rupprecht et al., 1996), they have additional targets of actions in neuronal cells, including cell surface neurotransmitter receptors (Rupprecht and Holsboer, 1999).

Pregnenolone, one of the most abundant neuroactive steroids, is a main precursor of steroid hormones synthe-

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sized from cholesterol. Its endogenous sulfated derivative, pregnenolone sulfate, exhibits diverse effects on ligand-gated ion channels, i.e., acting as a negative modulator of $GABA_A$ receptor channels (Lambert et al., 1995), while positively modulating NMDA receptor channels (Wu and Gibbs, 1991). Pregnenolone sulfate and synthetic derivatives such as pregnenolone hemisuccinate have also been reported to augment NMDA-induced increases in intracellular Ca^{2+} in cultured rat hippocampal neurons (Irwin et al., 1994). Moreover, behavioral studies have shown that pregnenolone sulfate increases the convulsant potency of NMDA (Maione et al., 1992). In contrast, pregnanolone sulfate (3α-hydroxy-5β-pregnan-20-one sulfate; 3α5βS), a metabolite of progesterone, inhibits NMDA-evoked membrane current and NMDA-induced increase in intracellular Ca^{2+} (Park-Chung et al., 1997). Other neuroactive steroids of particular interest are dehydroepiandrosterone and dehydroepiandrosterone sulfate, since they were reported to protect hippocampal neurons against exitotoxicity (Kimonides et al., 1998). Despite those studies investigating the effects of neuroactive steroids, little is known about the contribution of direct modulation by neuroactive steroids of NMDA receptors to their regulatory actions on excitotoxic neuronal death.

In the present study, we investigated the effects of various steroidal compounds on NMDA receptor-mediated excitotoxic cell death in organotypic cerebrocortical slice cultures. Moreover, we examined potential mechanisms of actions of 3α5βS, pregnenolone sulfate and pregnenolone hemisuccinate, which showed marked influences on NMDA neurotoxicity.

2. Materials and methods

2.1. Drugs and chemicals

Unless otherwise indicated, drugs and chemicals were obtained from Nacalai Tesque (Kyoto, Japan). Corticosterone, dehydroepiandrosterone, dehydroepiandrosterone sulfate, pregnenolone, (5*R*, 10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]-cycloheptan-5,10-imine maleate (MK-801 maleate), forskolin, bisindolylmaleimide and phorbol-12-myristate-13-acetate (PMA) were obtained from Sigma (St. Louis, MO, USA). 3α5βS, pregnenolone sulfate and pregnenolone hemisuccinate were purchased from Steraloids (Newport, RI, USA). Cycloheximide and dibutyryl cyclic AMP (db-cAMP) were from Wako (Osaka, Japan).

Cycloheximide, bisindlylmaleimide, MK-801 were dissolved in sterile purified water and added to the medium at 1000-fold dilution. The solution of dibutyryl cyclic AMP was prepared in serum-free medium at 10 mM, diluted at 10-fold before use. The stock solution of NMDA was prepared at 50 mM in water. All other drugs were dissolved in dimethyl sulfoxide and added to the medium or buffer at 1000-fold dilution. Control group in all sets of experiments received treatment with the corresponding vehicle (0.1% water or 0.1% dimethyl sulfoxide). Dimethyl sulfoxide at this concentration had no effect on NMDA neurotoxicity, NMDA-induced currents or changes in intracellular Ca^{2+} levels.

2.2. Cortical slice cultures

All animals used in the present study were treated in accordance with the guidelines of Kyoto University animal experimentation committee, and the guidelines of the Japanese Pharmacological Society. Organotypic slice cultures were prepared essentially according to the methods described previously (Katsuki et al., 2001b). Briefly, postnatal days 2–3 Wistar rats (Nihon SLC, Shizuoka, Japan) were anesthetized by hypothermia, brains were removed from the skull and separated into two hemispheres. Each hemisphere was cut into coronal slices of 300 μm thickness under sterile conditions, using a tissue chopper (Narishige, Tokyo, Japan). Slices containing the parietal cortex and the striatum were chosen, and other brain structures such as the septum and the basal forebrain were removed from each slice. Six corticostriatal tissue slices were transferred onto a 30-mm Millicell-CM insert membrane (with pore size of 0.4 μm, Millipore, Bedford, MA, USA) in six-well plates (Corning Costar, Tokyo, Japan). Culture medium, consisting of 50% minimal essential medium/HEPES (GIBCO, Invitrogen Japan, Tokyo Japan), 25% Hank's balanced salt solution (GIBCO) and 25% heat-inactivated horse serum (GIBCO) supplemented with 6.5 mg/ml glucose and 2 mM L-glutamine, 100 U/ml penicillin G potassium and 100 μg/ml streptomycin sulfate (GIBCO), was supplied at 0.7 ml/well. Culture medium was replaced with fresh medium on the next day of culture preparation, and thereafter, every other day. Slices were cultured for 10–11 days in a humidified atmosphere of 5% CO_2 and 95% air at 34 °C.

Twenty-four hours before application of NMDA, culture medium was replaced with serum-free medium, which consisted of 75% minimal essential medium/HEPES and 25% Hank's balanced salt solution supplemented with 6.5 mg/ml glucose, 2 mM L-glutamine, 100 U/ml penicillin G potassium and 100 μg/ml streptomycin sulfate. Subsequent treatment with NMDA for 24 h was done with serum-free medium of the same composition. Drugs were concomitantly applied with NMDA, unless otherwise noted.

Cortical neurons in slice cultures were identified by Nissl staining with toluidine blue (Chroma, Kongen, Germany). After drug treatment, cultures were fixed with 0.1 M phosphate buffer containing 4% paraformaldehyde and 4% sucrose overnight, rinsed with distilled water twice and exposed to 0.1% toluidine blue solution for 20 min. Following a further rinse in distilled water, specimens were dehydrated through a graded series of ethanol (70%, 96% and 100%) and mounted with glycerol on slide glasses.

Positively stained cells with round or oval cell bodies and distinct nuclear boundaries were considered as viable cortical neurons. The maximal number of surviving neurons in an area of 80×90 μm^2 within the parietal cortex of individual slices was counted. Reproducibility of the results was confirmed by at least two different sets of experiments. Data shown in figures are from a representative set of experiments.

2.3. Dissociated cortical cultures

Primary cultures of dissociated cortical neurons were obtained from the cerebral cortex of fetal Wistar rats (17–19 days of gestation) according to the procedures described previously (Nishikawa et al., 2000). Briefly, single cells mechanically dissociated from the whole cerebral cortex were plated on plastic coverslips coated with polyethylenimine, which were placed in Falcon 60-mm dishes (5.1×10^6 cells per dish). Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere in Eagle's minimal essential medium (Nissui Pharmaceuticals, Tokyo, Japan) supplemented with glutamine (2 mM), glucose (total 11 mM), $NaHCO_3$ (24 mM), HEPES (10 mM) and 10% heat-inactivated fetal bovine serum (1–7 days in vitro; JRH Biosciences, Lenexa, KS, USA) or 10% heat-inactivated horse serum (8–12 days in vitro; JRH Biosciences). Six days after plating, nonneuronal cells were removed by addition of 10 μM cytosine arabinoside (Sigma).

In neurotoxicity experiments, cultures at 11–12 days in vitro were incubated in serum-free Eagle's minimal essential medium for 1 h at 37 °C containing glutamate and drugs, then stained with 1.5% trypan blue solution for 10 min, fixed with 10% isotonic formalin, and rinsed with physiological saline. Cells stained with trypan blue were regarded as nonviable, and unstained cells were regarded as viable. The viability of the cultures was evaluated by the percentage of unstained cells relative to the total number of cells.

2.4. Electrophysiology

Whole-cell currents were recorded from dissociated cortical neurons at 11–12 days in vitro using the standard patch-clamp techniques (Kawahara et al., 1994; Kume et al., 2002). Glass patch electrodes (tip resistance of 4–7 MΩ) were filled with the internal solution composed of 145 mM CsCl, 11 mM EGTA, 4 mM Mg-ATP and 10 mM HEPES (pH adjusted to 7.2 with CsOH). The recording chamber was perfused continuously at a rate of 2–3 ml/min with the external solution (145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 10 mM HEPES, 10 mM D-glucose, 10 μM glycine, pH adjusted to 7.4 with NaOH). Drug application was done using a gravity-driven external perfusion system of an array of seven capillary tubes (280-μm inner diameter) placed near the cell. Currents were recorded at room temperature (21–25 °C) using the EPC9 patch clamp amplifier (HEKA, Lambrecht, Germany). Series resistance compensation was not routinely used.

2.5. Measurements of intracellular Ca^{2+} concentrations

Glutamate-evoked increases in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) were measured with a Ca^{2+}-sensitive fluorescent dye, fura-2 acetoxymethyl ester (Dojindo, Kumamoto, Japan), using a fluorescence imaging system (ARGUS-HiSCA, Hamamatsu Photonics, Shizuoka, Japan) equipped with a cooled CCD camera. Dissociated cortical neurons cultured on polyethylenimine-coated cover glasses (13 mm in diameter, Matsunami Glass, Japan) at 11–12 days in vitro were incubated in Krebs–Ringer buffer (137 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM HEPES, 25 mM D-glucose, pH adjusted to 7.4) containing 5 μM fura-2 acetoxymethyl ester and 0.01% cremophore EL (Sigma) for 30 min at 37 °C. After postincubation for at least 30 min, a cover glass was transferred to a recording chamber (500 μl in volume) settled on the stage of an inverted fluorescence microscope (TE300, Nikon). Fura-2 fluorescence obtained by excitation wavelengths of 340 and 380 nm was recorded every 2 s. After stable recordings were obtained, drug solutions were applied to the recording chamber to give final concentrations of drugs as indicated in Results.

2.6. Statistical analysis

Data are expressed as means ± S.E.M. Statistical significance of differences was evaluated with one-way analysis of variance followed by Student–Newman–Keuls' test for neurotoxicity experiments on slice cultures, and by Dunnett's two-tailed test for experiments of intracellular Ca^{2+} measurements. Data of electrophysiological experiments were evaluated by paired *t*-test. Probability values less than 5% were considered significant.

3. Results

3.1. Effects of steroidal compounds on NMDA-induced neuronal cell death in cortical slice cultures

Cultured cortical slices at 11–12 days in vitro maintained well-organized cellular architectures of the cerebral cortex. Individual neurons within the cortical area could be clearly visualized under a light microscope after Nissl staining (Fig. 1A). Application of NMDA (3–100 μM) to the slice cultures for 24 h caused a concentration-dependent reduction in the number of viable cortical neurons (Fig. 1B). NMDA neurotoxicity displayed very steep concentration–response relationship at concentrations between 10 and 30 μM. Accordingly, the degree of cell death induced by NMDA at intermediate concentrations such as 15 and 20 μM sometimes varied among different sets of experiments

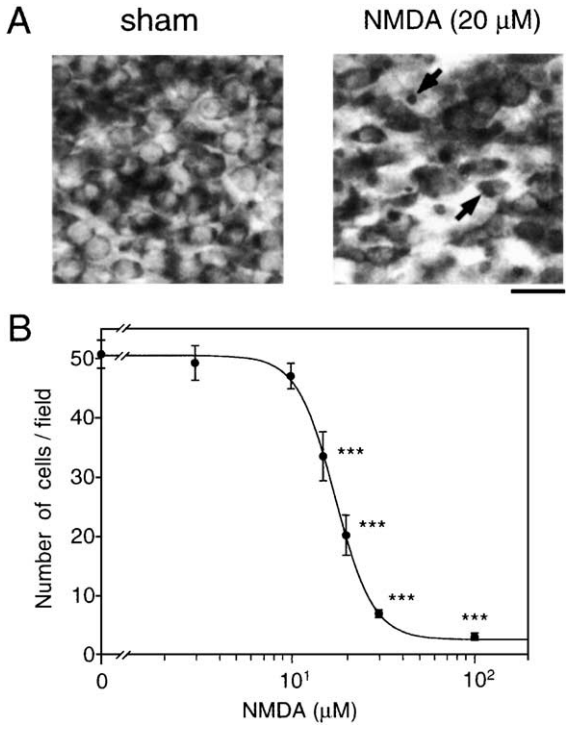

Fig. 1. Neuronal death induced by NMDA in rat cortical slice cultures. Slices at 11 days in vitro were exposed to NMDA (0–100 μM) for 24 h. (A) Photomicrographs of Nissl staining of cortical cultures that received indicated treatments. Note the appearance of pyknotic cells in NMDA-treated culture (arrows). Scale bar = 20 μm. (B) Summary of the data showing the effect of NMDA on the number of surviving neurons. $n = 11–12$ for each point. ***$P < 0.001$ compared with sham (0 μM NMDA).

(for example, see Fig. 2). We confirmed, however, that the number of surviving cells did not vary when the same sister cultures were exposed to the same concentration of NMDA. Simultaneous application of MK-801 (1 μM), a noncompetitive antagonist of the NMDA receptor channel complex, virtually abolished the neurotoxic effect of NMDA (data not shown).

We examined the effects of eight kinds of steroidal compounds on NMDA-induced neuronal death in cortical slice cultures. Concurrent application of representative neurosteroids such as pregnenolone, dehydroepiandrosterone and dehydroepiandrosterone sulfate with 15 or 20 μM NMDA did not influence the extent of NMDA-induced cell death. These neurosteroids exerted no significant effects on NMDA neurotoxicity even when they were applied from 24 h before NMDA application (Fig. 2A). Similarly, corticosterone and prednisolone did not affect NMDA-induced neuronal death, even at a concentration as high as 100 μM (Fig. 2A). On the other hand, concurrent application of 3α5βS (30–300 μM) with 20 μM NMDA resulted in a marked, concentration-dependent suppression of NMDA neurotoxicity (Fig. 2B). In a sharp contrast to the results with 3α5βS, pregnenolone sulfate (30–300 μM) and pregnenolone hemisuccinate (10–100 μM) clearly exacerbated NMDA-induced death of cortical neurons in a concentration-dependent manner, when they were co-applied with 15 μM NMDA (Fig. 2B). In the following experiments, therefore, we further examined the actions of 3α5βS, pregnenolone sulfate and pregnenolone hemisuccinate on NMDA-induced neuronal death.

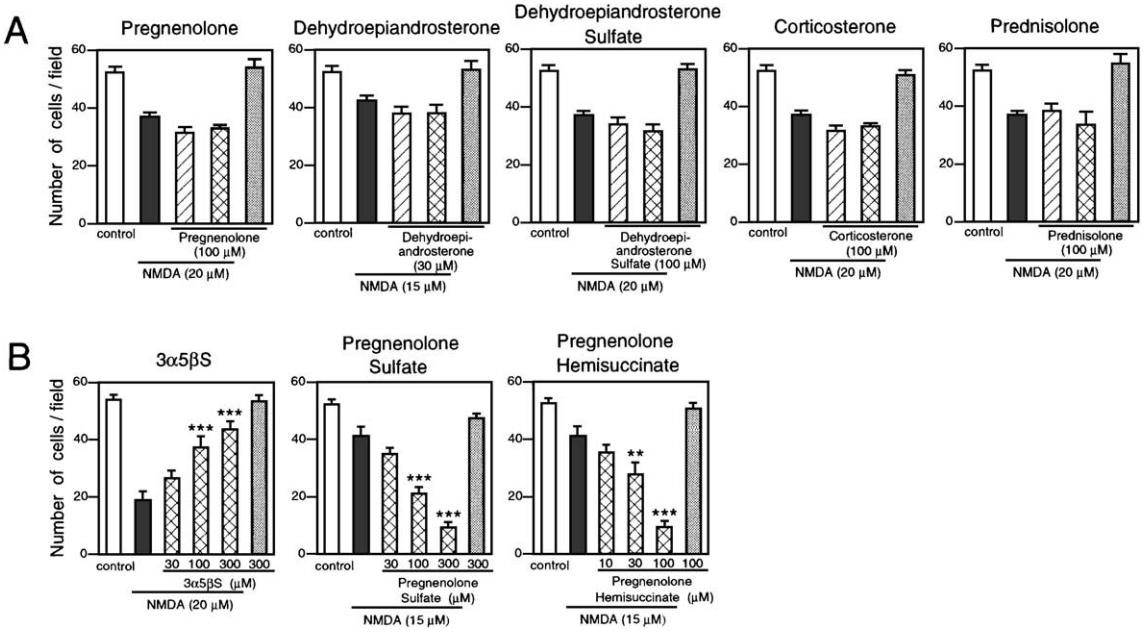

Fig. 2. Effects of eight steroidal compounds on NMDA-induced neuronal death in cortical slice cultures. Steroids were pre-administered for 24 h and co-administered with NMDA for subsequent 24 h (hatched column), or only simultaneously applied with NMDA for 24 h (cross-hatched column). Five steroids in panel A showed no effects, whereas 3α5βS, pregnenolone sulfate and pregnenolone hemisuccinate in panel B exerted concentration-dependent effects on NMDA-induced neuronal death. $n = 5–6$ for each column in panels A, and $n = 11–12$ for each column in panels B. **$P < 0.01$, ***$P < 0.001$ compared with NMDA alone.

3.2. Assessment of potential sites of actions of neuroactive steroids

Several neuroactive steroids have been reported to express their biological activities through their binding to specific intracellular receptors and initiation of new protein synthesis (Rupprecht et al., 1996). To determine whether de novo protein synthesis was involved in the modulatory actions of the above-mentioned steroids on NMDA-induced cell death, we examined the effect of cycloheximide, an inhibitor of protein synthesis (Furukawa et al., 1997). In this set of experiments, cycloheximide at a concentration of 0.1 μg/ml was applied concurrently with 3α5βS (300 μM), pregnenolone sulfate (100 μM) or pregnenolone hemisuccinate (30 μM) from 24 h before the application of NMDA, and present in the culture medium with steroids during entire period of NMDA exposure. We recently demonstrated that this concentration of cycloheximide significantly affected the in vitro survival of retinal ganglion cells (Manabe et al., in press). Consistent with the above results, 3α5βS inhibited, whereas pregnenolone sulfate and pregnenolone hemisuccinate exacerbated, NMDA-induced cell death. The effects of these steroids were also observed in the presence of cycloheximide, virtually to the same extent to those observed in the absence of this protein synthesis inhibitor (Fig. 3A). These results suggest that protein synthesis is not required for the effects of neuroactive steroids on NMDA neurotoxicity.

Several species of neuroactive steroids are shown to modulate the activities of $GABA_A$ receptor channels (Lambert et al., 1995; Park-Chung et al., 1999). This action may be important with respect to the regulation of excitotoxicity, because previous reports have demonstrated that GABA exerts a protective action against exitotoxicity in cultured cortical neurons (Muir et al., 1996; Ohkuma et al., 1994; but see Yoshikawa et al., 1998). Therefore, we examined the effect of a $GABA_A$ receptor antagonist picrotoxin. We confirmed in electrophysiological experiments that picrotoxin at 30 μM markedly attenuated GABA-induced current in dissociated cortical cultures (data not shown). However, concurrent application of picrotoxin (100 μM) with NMDA did not modify NMDA-induced cell death in cortical slice cultures. Moreover, the regulatory actions of 3α5βS, pregnenolone sulfate and pregnenolone hemisuccinate on NMDA neurotoxicity were not affected by the presence of picrotoxin (Fig. 3B).

In another set of experiments, we examined the effects of neuroactive steroids on NMDA-induced cytotoxicity in the presence of a σ receptor antagonist rimcazole (Kennedy et al., 1990), because neuroactive steroids may exert some of their biological effects by acting as σ receptor ligands (Bergeron et al., 1996; Monnet et al., 1995).

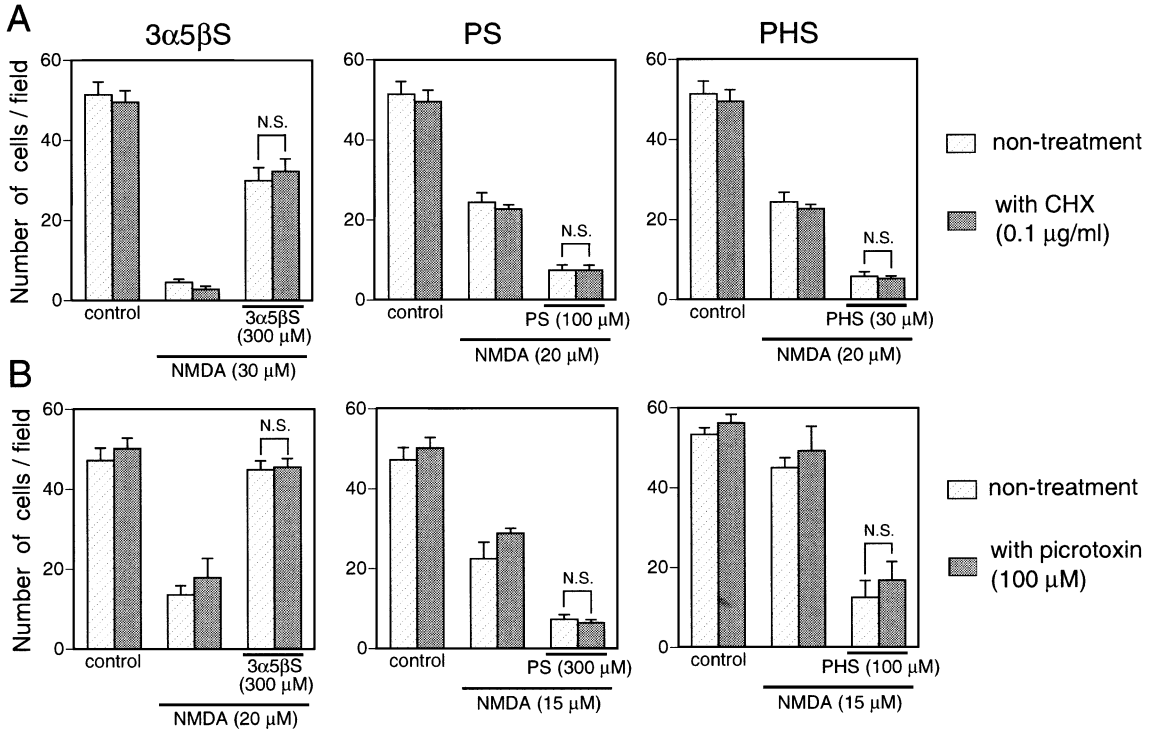

Fig. 3. Cycloheximide (CHX; A) and picrotoxin (B) do not affect the modulatory actions of 3α5βS, pregnenolone sulfate and pregnenolone hemisuccinate on NMDA-induced neuronal death in cortical slice cultures. In A, steroids and CHX (0.1 μg/ml) were pre-applied for 24 h, and co-applied with NMDA for subsequent 24 h. In B, steroids and picrotoxin (100 μM) were concurrently applied with NMDA for 24 h. $n = 5–6$ for each column. N.S.: not significant.

However, rimcazole (10 μM) did not exhibit significant influences on the regulatory actions of 3α5βS, pregnenolone sulfate and pregnenolone hemisuccinate on NMDA neurotoxicity (data not shown). Altogether, these results exclude possible involvement of some well-known sites of actions of neuroactive steroids, and therefore, imply that direct modulation of NMDA receptor channels plays a crucial role in the regulation of excitotoxicity by these steroids.

3.3. Protein kinases are not involved in the effect of neuroactive steroids

NMDA receptor functions are regulated by protein kinases such as protein kinase A (PKA) and protein kinase C (PKC) (Greengard et al., 1991; Lan et al., 2001). Moreover, some of the effects of neuroactive steroids on neurotransmitter receptor-mediated responses are shown to be dependent on protein phosphorylation (Fancsik et al., 2000; Gu and Moss, 1996). Accordingly, we examined whether manipulation of the activities of PKA or PKC alters the observed actions of neuroactive steroids on NMDA neurotoxicity. We tested the effects of forskolin, an adenylyl cyclase activator, and db-cAMP, a membrane-permeable cyclic AMP analog. We previously demonstrated that these drugs remarkably affected the maintenance of dopaminergic neurons in midbrain slice cultures (Katsuki et al., 2001a). Forskolin (20 μM) or db-cAMP (1 mM) applied simultaneously with NMDA for 24 h did not exert any influences on NMDA neurotoxicity. In addition, the effects of 3α5βS, pregnenolone sulfate and pregnenolone hemisuccinate on NMDA neurotoxicity were not affected by these drugs (Fig. 4A). We also examined the effects of PMA, a PKC activator, and bisindolylmaleimide, a selective PKC inhibitor (Fancsik et al., 2000). In a similar manner to those observed with forskolin and db-cAMP, PMA (100 nM) and bisindolylmaleimide (500 nM) did not show any significant influences either on NMDA-induced cell death or on the regulatory actions of neuroactive steroids on NMDA neurotoxicity (Fig 4B).

3.4. Regulation of NMDA receptor-mediated currents by neuroactive steroids

In the next set of experiments, we investigated the effects of neuroactive steroids on NMDA receptor-mediated currents in cortical neurons, using whole-cell patch clamp recording. For this purpose, we employed dissociated primary neuronal cultures prepared from fetal rat cerebral cortex and maintained in vitro for 11–12 days. We confirmed that three neuroactive steroids markedly affected glutamate-induced neuronal death in this preparation. That is, 3α5βS (10–100 μM) inhibited, whereas

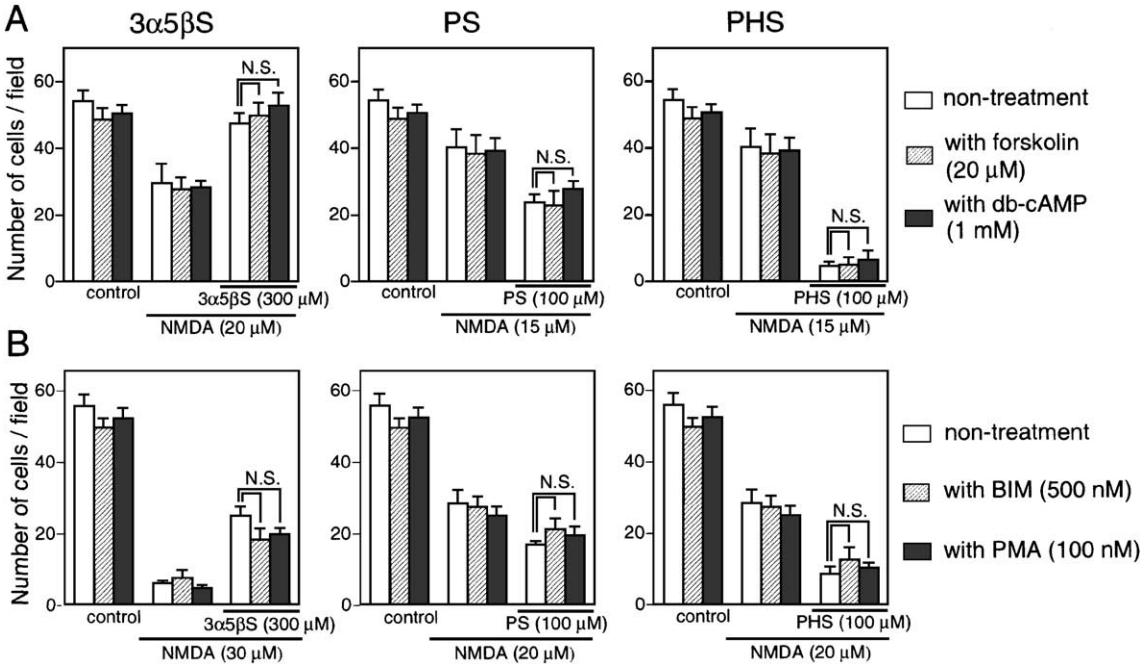

Fig. 4. Modulatory actions of 3α5βS, pregnenolone and pregnenolone hemisuccinate on NMDA-induced neuronal death were not affected by manipulation of cyclic AMP levels or protein kinase C activity. Effects of forskolin (20 μM) and db-cAMP (1 mM) are shown in A, and effects of bisindolylmaleimide (BIM; 500 nM) and PMA (100 nM) are shown in B. Steroids and drugs were simultaneously applied with NMDA for 24 h. $n = 5–6$ for each column. N.S.: not significant.

pregnenolone sulfate (10–100 μM) and pregnenolone hemisuccinate (10–100 μM) exacerbated, glutamate-induced death in a concentration-dependent manner (data not shown).

Whole-cell patch clamp recordings revealed that application of NMDA (3–1000 μM) to cortical neurons induced a robust inward current at a holding potential of −60 mV, in a concentration-dependent manner (Fig. 5A). We obtained virtually the same amplitude of current responses repeatedly from a single neuron, when we set the duration and interval of application of 30 μM NMDA to 10 s and 1 min, respectively. 3α5βS (100 μM), pre-administered for 10 s and co-applied with 30 μM NMDA for another 10 s, significantly reduced whole-cell inward current induced by NMDA (Fig. 5B and C). The effect of 3α5βS was reversible, and the amplitude of the current response to the next challenge with NMDA in the absence of 3α5βS almost returned to the baseline level (Fig. 5C). On the other hand, pregnenolone hemisuccinate (100 μM) applied in the same manner significantly potentiated the current responses of cortical neurons to 30 μM NMDA, which was reversible after washout. Interestingly, pregnenolone sulfate (100 μM) did not augment NMDA-induced inward current responses (Fig. 5B and C), despite the fact that this compound markedly exacerbates NMDA and glutamate neurotoxicity.

3.5. Glutamate-induced increases in intracellular Ca^{2+} are modulated by neuroactive steroids

Excessive increase in cytoplasmic Ca^{2+} levels is considered to be a primary trigger of glutamate-induced neuronal death. Therefore, we evaluated the effects of neuroactive steroids on glutamate-induced changes in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in dissociated cortical neurons, using fura-2 fluorescence imaging. Application of 100 μM glutamate resulted in a prompt increase in $[Ca^{2+}]_i$, which gradually declined over 9 min of observation period. Consistent with its neuroprotective effect, application of 3α5βS (300 μM) markedly attenuated glutamate-induced $[Ca^{2+}]_i$ increase (Fig. 6). On the other hand, pregnenolone hemisuccinate (100 μM) augmented the peak amplitude of $[Ca^{2+}]_i$ increase induced by glutamate, which is also consistent with the fact that this compound exacerbates NMDA and glutamate neurotoxicity in cortical neurons. In addition to augmenting the peak amplitude of $[Ca^{2+}]_i$ response, pregnenolone hemisuccinate prevented the decay of $[Ca^{2+}]_i$ increase over the entire observation period. This effect of pregnenolone hemisuccinate was reflected in the evaluation of the area under the curve of time-dependent changes in $[Ca^{2+}]_i$, where augmentation of $[Ca^{2+}]_i$ response by pregnenolone hemisuccinate was more robustly detected than in the peak amplitude of $[Ca^{2+}]_i$ increases (Fig. 6B).

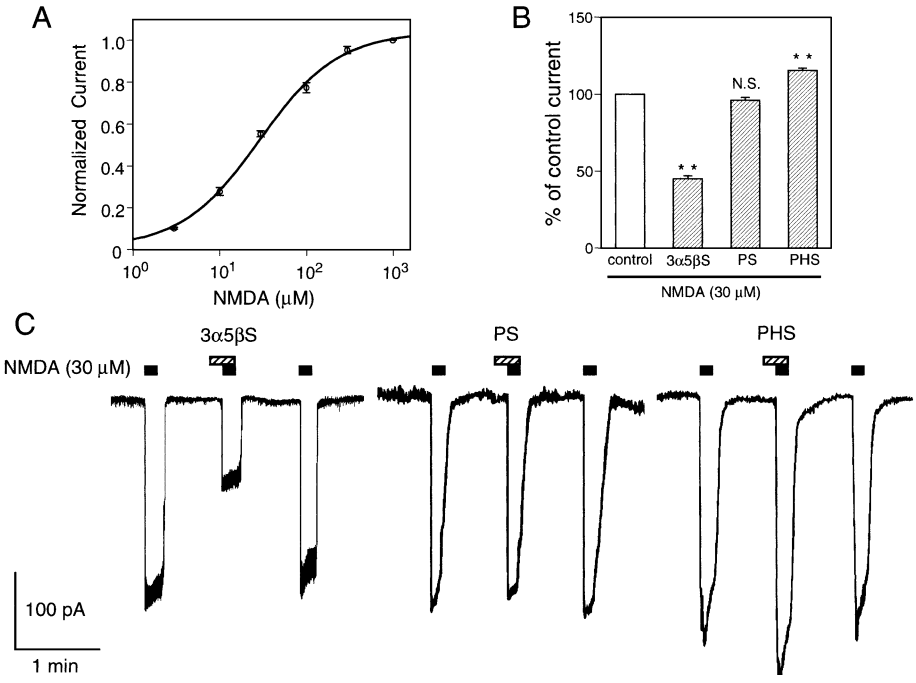

Fig. 5. Modulatory actions of neuroactive steroids on NMDA-induced whole-cell currents in dissociated cortical neurons. (A) Concentration dependency of the amplitude of current responses evoked by application of the indicated concentrations of NMDA. $n=8$ for each data point. (B) Summarized data showing the effects of 3α5βS, pregnenolone and pregnenolone hemisuccinate on NMDA-evoked currents. Steroids at a concentration of 100 μM were pre-applied for 10 s and co-applied with NMDA for subsequent 10 s. $n=42$ for each column. ** $P<0.01$ compared with NMDA alone; N.S.: not significant. Representative traces of current responses to NMDA in the absence and presence of each steroid are shown in C.

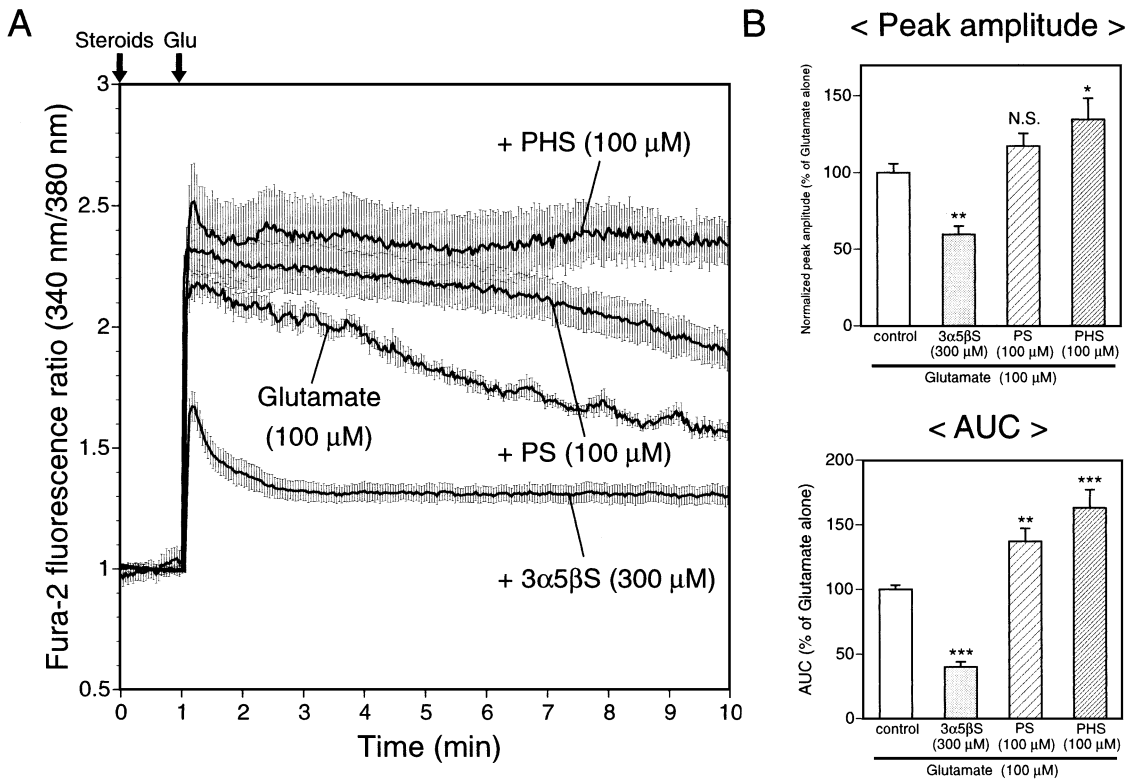

Fig. 6. Effects of 3α5βS, pregnenolone sulfate and pregnenolone hemisuccinate on glutamate-evoked increases in $[Ca^{2+}]_i$ in dissociated cortical cultures. (A) Time course of changes in fura-2 fluorescence ratio. Steroids were present in extracellular fluid over the entire period of recording, and glutamate (Glu) at a final concentration of 100 μM was added to the extracellular fluid at 1 min, as indicated by arrows at the top of the panel. (B) Peak amplitude of $[Ca^{2+}]_i$ increase was determined from the maximal value of fluorescence ratio just after the addition of glutamate. Area under the $[Ca^{2+}]_i$ curve (AUC) was estimated by the area delineated by fluorescence ratio values from 1 to 10 min. $n=6$ for each treatment group. Reproducibility was confirmed by two different sets of experiments on different sister cultures. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with control; N.S.: not significant.

Notably, pregnenolone sulfate showed a qualitatively similar effect to that of pregnenolone hemisuccinate, although to a lesser extent. Whereas pregnenolone sulfate (100 μM) exhibited a slight and nonsignificant tendency to increase the peak amplitude of $[Ca^{2+}]_i$ responses, this compound slowed the decay of $[Ca^{2+}]_i$ increase seen after application of 100 μM glutamate, and significantly increased the area under the curve of time-dependent changes in $[Ca^{2+}]_i$ (Fig. 6B).

4. Discussion

We demonstrated in the present study that neuroactive steroids markedly affect NMDA-induced neuronal cell death in rat cortical neurons. Among eight steroidal compounds tested, 3α5βS inhibited, whereas pregnenolone sulfate and pregnenolone hemisuccinate exacerbated, NMDA neurotoxicity in a concentration-dependent manner. These results are consistent with previous reports showing the effects of neuroactive steroids using different preparations and experimental paradigm. For example, 3α5βS was neuroprotective against NMDA excitotoxicity in rat hippocampal cultures (Weaver et al., 1997). Pregnanolone hemisuccinate, a synthetic analog of 3α5βS, reduced ischemic damage in rat brain following middle cerebral artery occlusion, and antagonized NMDA-induced seizures (Weaver et al., 1997). In addition, pregnenolone sulfate has been shown to exacerbate NMDA-induced death of hippocampal neurons (Weaver et al., 1998). On the other hand, we did not observe neuroprotective effects of dehydroepiandrosterone and dehydroepiandrosterone sulfate, both of which have been reported to protect hippocampal neurons from excitotoxic insults (Kimonides et al., 1998). Although the reasons for discrepancy are not clear, the neuroprotective effects of dehydroepiandrosterone and dehydroepiandrosterone sulfate observed by Kimonides et al. (1999) may be exerted through the interactions of these steroids with steroid hormone receptor-related events, which might not be active in cortical neurons in our preparations.

Whereas pregnenolone sulfate and pregnenolone hemisuccinate markedly augmented NMDA cytotoxicity, pregnenolone, one of the principal neuroactive steroids in the brain, did not exert any influences on NMDA-induced neuronal death. Given the fact that augmentation of excitotoxicity by pregnenolone sulfate and pregnenolone hemisuccinate resulted from the enhancement of activities of NMDA receptor-associated channels (as discussed below),

these results are consistent with reports demonstrating that a negative charge and configuration of substituents at the C3 position, and geometric structure at the C5–C6 position is an important determinant of the modulatory activity of neuroactive steroids on NMDA receptors (Irwin et al., 1994; Weaver et al., 2000).

To date, the mechanisms of cytoprotective and/or death-promoting actions of steroidal compounds have not been pursued in detail, and in the present study, we addressed this issue by examining possible roles of several established sites of actions of neuroactive steroids. These include (1) initiation of de novo protein synthesis following activation of intracellular steroid hormone receptors, (2) regulation of the activities of $GABA_A$ receptor-associated channels, and (3) interaction with σ receptors located mainly on endoplasmic reticulum, where they may regulate Ca^{2+} release from internal stores (Hayashi and Su, 2001). However, pharmacological interventions in these pathways all failed to alter the actions of 3α5βS, pregnenolone sulfate and pregnenolone hemisuccinate on NMDA-induced neuronal death in cortical slice cultures. These results led us to assume that, among other potential sites of action, NMDA receptor-associated channels themselves are primarily involved in the regulatory effects of these neuroactive steroids on excitotoxicity.

Activities of NMDA receptor-associated channels are positively regulated by phosphorylation of receptor subunit proteins by PKA and PKC (Greengard et al., 1991; Lan et al., 2001). These protein kinases are also implicated in biological actions of several neuroactive steroids. For example, potentiation of kainate-induced currents by 17β-estradiol in hippocampal neurons is blocked by a specific inhibitor of PKA (Gu and Moss, 1996). In addition, allopregnanolone potentiates GABA-mediated inhibitory synaptic currents in hypothalamic magnocellular neurons, which is attenuated by inhibitors of PKC as well as of PKA (Fancsik et al., 2000). Taking these observations into account, we tested the effects of several compounds expected to alter the activities of PKA and PKC. However, we did not detect any influences of manipulation of cyclic AMP levels and PKC activity on the actions of 3α5βS, pregnenolone sulfate and pregnenolone hemisuccinate. These results suggest that the regulatory actions of neuroactive steroids on excitotoxicity are independent of cellular kinase activity and protein phosphorylation levels.

Accordingly, we employed electrophysiological examination of whole-cell current responses and microfluorometric examination of $[Ca^{2+}]_i$ changes, to reveal the relationship between regulation of NMDA receptor channels and regulation of excitotoxicity by neuroactive steroids. These measurements clearly revealed that 3α5βS inhibits, whereas pregnenolone hemisuccinate enhances, the activity of NMDA receptor-associated channels, causing attenuation and enhancement, respectively, of glutamate-induced increase in $[Ca^{2+}]_i$. These results are in line with previous reports showing that 3α5βS negatively and pregnenolone hemisuccinate positively regulate functions of NMDA receptor channels in cultured rat hippocampal neurons (Irwin et al., 1994; Weaver et al., 2000). They are also consistent with the findings in the present study that 3α5βS inhibits and pregnenolone hemisuccinate exacerbates NMDA neurotoxicity in cortical slice cultures and glutamate neurotoxicity in dissociated cortical neurons. A notable finding is that pregnenolone sulfate did not exhibit appreciable influences on NMDA-induced current responses in cultured cortical neurons, and we could detect enhancement of NMDA receptor functions by pregnenolone sulfate only when we monitored $[Ca^{2+}]_i$ changes following prolonged application of glutamate and pregnenolone sulfate. These observations were unexpected, because pregnenolone sulfate has been reported to markedly enhance currents through heterologously expressed NMDA receptor channels in *Xenopus* oocytes as well as native NMDA receptor channels in chick spinal cord and rat hippocampal neurons (Park-Chung et al., 1997). In this context, a recent study reported that pregnenolone sulfate exhibits both stimulatory and inhibitory effects on NMDA receptor channels, depending on the compositions of receptor subunits (Malayev et al., 2002). Therefore, relatively modest effects of pregnenolone sulfate on NMDA receptor functions in cortical neurons may be attributable to the properties of NMDA receptor channels expressed in these cells. It is also possible that, because NMDA receptor functions are strongly regulated by a variety of receptor-associated proteins (Scannevin and Huganir, 2000), differences in expression patterns of these associated proteins, rather than those of NMDA receptor subunits themselves, determine the potency of the effects of neuroactive steroids. Nevertheless, the enhancement of glutamate-induced increase in $[Ca^{2+}]_i$ by pregnenolone sulfate is consistent with the results that pregnenolone sulfate exacerbated NMDA and glutamate cytotoxicity on cortical neurons. In this context, a recent report provided evidence that pregnenolone sulfate may decrease agonist unbinding from NMDA receptors (Ceccon et al., 2001), which is expected to result in prolonged receptor activation and enhancement of Ca^{2+} influx.

Overall, the present study demonstrated that several neuroactive steroids regulate NMDA receptor-mediated glutamate excitotoxicity on cortical neurons, by directly modulating the activities of NMDA receptor-associated channels, thereby regulating Ca^{2+} influx into neurons. The concentrations of 3α5βS and pregnenolone sulfate required to influence excitotoxic consequences in cortical neurons were over 30 μM, which were well above the reported concentrations of neuroactive steroids in adult rat brain (Baulieu, 1997) and in human plasma (Pearson-Murphy et al., 2000). It is conceivable, however, that the levels of neuroactive ring A-reduced metabolites of progesterone in human plasma during pregnancy are elevated to a level 10–1000 times higher than normal (Pearson-Murphy et al., 2000). Moreover, neuroactive steroids are synthesized and released within the brain, and considerably higher concentrations

may be achieved locally under pathological conditions including cerebral ischemia, epilepsy and depression (Di Michele et al., 2000; Rupprecht and Holsboer, 1999). The observed effects of neuroactive steroids may be involved in pathophysiological processes in the brain as part of endogenous regulatory system.

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