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Title:

β-Estradiol, DHEA and DHEA-S protect against NMDA-induced neurotoxicity in rat hippocampal neurons by different mechanisms

Authors:

KENICHI KURATA, MINORU TAKEBAYASHI, SHIGERU MORINOBU and SHIGETO YAMAWAKI

Affiliation:

Department of Psychiatry and Neurosciences, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Japan. (K.K., S.M., S.Y.)

Laboratory of Psycho-oncology, Institute of Clinical Research, National Hospital Organization Kure Medical Center, Japan. (M.T.)

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Running title: Neuroprotective effects of β-estradiol, DHEA and DHEA-S

Corresponding author: Shigeto Yamawaki, M.D., Ph.D., Department of Psychiatry and Neurosciences, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. Tel: (81) (82)-257-5207. Fax: (81) (82)-257-5209, e-mail address: yamawaki@hiroshima-u.ac.jp (S.Yamawaki).

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ABBREVIATIONS: DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate; NMDA, N-methyl-D-aspartate; NO, nitric oxide; NOS, nitric oxide synthase; EAAs, excitatory amino acids; CNS, central nervous system; ANOVA, analysis of variance; NMDA-R, N-methyl-D-aspartate receptor; GABA-A-R, γ-aminobutyric acid type A receptors; Sig-1R, sigma-1 receptors; Sig-2R, sigma-2 receptor; VGCCs, voltage-gated calcium channels; NO_2^-, nitrite; NO_3^-, nitrate; ROS, reactive oxygen species; $[Ca^{2+}]i$, intracellular calcium concentration; PKC, protein kinase C; BSS, balanced salt solution; SNP, sodium nitroprusside; L-NMMA, N^G-Monomethyl-L-arginine acetate; BD1063, 1-[2-(3,4-Dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride.

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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 co-treatment 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. A NOS inhibitor, L-NMMA 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 Sig-1R antagonists rimcazole or BD-1063 partially, but significantly, reversed the neuroprotective effect of DHEA-S against NMDA-induced neurotoxicity, while muscimol, a GABA-A-R agonist, did not. This suggests that the neuroprotective effect of DHEA-S may be mediated via Sig-1R, at least in part. Taken together, our data suggest that the neurosteroid family members β-estradiol, DHEA and DHEA-S exert neuroprotective effects through different non-genomic mechanisms.

INTRODUCTION

Stress induces an elevation of excitatory amino acids (EAAs) in the brain including in the prefrontal cortex, hippocampus and basal ganglia (Moghaddam et al., 1993). EAAs 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 et al., 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).

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

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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 non-genomic 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 sigma-1 receptors (Sig-1R) (Majewska et al., 1990; Monnet et al., 1995; Kurata et al., 2001). Although a large number of studies has 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 in order to better elucidate mechanisms of neuroprotection.

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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 (Bristol, UK); β-estradiol, DHEA, DHEA-S, muscimol, rimcazole, poly-L-lysine, deoxyribonuclease and nifedipine from Sigma Chemical Co. (St. Louis, MO); MK801 from Research Biochemicals (MA, USA); 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 from Gibco (Grand Island, USA); DMSO from Wako (Osaka, Japan); penicillin and streptomycin 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, 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℃ 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 - 3 more days. This replacement is appropriate to elucidate effects of hormones against excitatory amino acid-induced neurotoxicity because N2 growth medium contains less hormones and anti-oxidants than B27 growth medium. On the 10 - 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 Fig2A [1, 2, 3], after the NMDA exposure, cells were washed once with the neurobasal medium (without NMDA and steroid) and

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incubated for an additional period of 24 h under standard culture conditions before measurements. In Fig 2A [2] ("pre-treatment"), after the pre-treatment 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. As for Sig-1R and GABA-A-R ligands as shown in the Table, they were added to the cultures 15 min prior to 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,

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which 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).

Intracellular Ca^{2+} measurements in single cells. Measurements of intracellular calcium concentration ($[Ca^{2+}]i$) by fluoromicroscopic determination of fura-2 were performed as previously described (Kurata et al., 2001). Briefly, hippocampal cells were rinsed twice with BSS and then incubated with 5 μM of fura-2 acetoxymethyl ester in BSS for 60 min at 37℃. Fura-2-loaded cells were perfused with BSS, warmed to 37℃, and examined at a flow rate of 1.5 ml/min on the stage of a fluorescence microscope-video camera system (C-2000; Hamamatsu Photonics, Hamamatsu, Japan). The fluorescence intensity of fura-2 was measured at excitation wavelengths of 340 nm and 380 nm and an emission wavelength of 510 nm. Fluorescence was recorded at 5 sec 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 $[Ca^{2+}]i$.

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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 - 60 min (4 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 were 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 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 ℃ 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 Griess reagent were 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 5 - 7 cells were used for analysis. The student's unpaired t-test was used to compare between the two groups in Fig. 1B, 3B, 4 and Table. Two-way analysis of variance (ANOVA) was also used in Fig. 3B. For multiple comparisons, data were analyzed by ANOVA followed by PLSD test in Fig. 1A, 2B, 3B, 3C, 5A, 5B, 6. A P value of < 0.05 was considered statistically significant. The correlation coefficient between Fig. 1A and Fig. 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. One to 60 μM β-estradiol and DHEA

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) like a reverse-U shaped curve. Maximum protective effects were obtained at the concentration of 60 μM for both β-estradiol and DHEA and 100 nM DHEA-S. These concentrations of steroids were used in subsequent experiments (Fig. 1B, 2B, 4, 5A, Table).

Sixty μM β-estradiol, 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 + 24h; "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 μM 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 Fig. 1B, 2B, 3C and Table, 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

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steroid treatment. The time course is shown in Fig. 2A. Sixty μM β-estradiol, 60 μM DHEA and 100 nM DHEA-S were added either 24 h before NMDA exposure ("pre-treatment" in Fig. 2A [2]), at the same time as NMDA exposure ("co-treatment" 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 co-treatment 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 sec 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.

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Therefore, 15 min after measuring the first response to NMDA, the cells were stimulated again for 30 sec 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 prior to 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} 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 - 60 μM DHEA and 10 - 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 correlation between the neuroprotective effects of β-estradiol (Fig. 1A) and inhibitory effects on NMDA-induced $[Ca^{2+}]i$ increases (Fig. 3B) (Pearson's correlation

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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 MK801 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 MK801, 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.

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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 (□). While co-treatment with 100 nM DHEA-S did not significantly affect NMDA-induced NO production at 60 min, co-treatment with 10 μM β-estradiol or 10 μM DHEA resulted in significant inhibition of NO production at 5 min, 15 min, 30 min 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 (iNOS) and endothelial NOS

(eNOS), are expressed in the CNS. Here, we measured the total NOS activity. Each of the steroids, a non-specific 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, 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 co-treatment 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. Further, 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 which 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 μ M 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,

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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 sigma receptor antagonists, rimcazole and BD1063, and a specific GABA-A-R agonist, muscimol. Rimcazole has high affinities both of Sig-1R and sigma-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 μM rimcazole, 1 μM BD1063 and 10 μM muscimol treatment did not affect cell viability. However, treatment with rimcazole and BD-1063, 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.

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 non-genomic actions modulating membrane receptor functions, including NMDA-R and VGCCs, within milliseconds to minutes (Moss et al., 1997), suggesting that non-genomic 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. Further, 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 – 72 h. Here, neuroprotective effects were mediated through mechanisms such as the upregulation 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). Pre-treatment with a high concentration of β-estradiol (60 μM) also failed to reveal any neuroprotective effects (Fig. 2B). We did not examine effects of pre-treatment with low concentrations of β-estradiol in this study. The results presented here suggest that the neuroprotective mechanisms operating with pre-treatment may differ depending on the concentrations of β-estradiol. It is important to investigate the effects of β-estradiol at higher concentrations because of the reasons as follows (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 (MacLlusky 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).

One to 60 μM DHEA showed significant neuroprotective effects against NMDA-induced neurotoxicity, while 1 to 30 μM DHEA did not inhibit the NMDA-induced $[Ca^{2+}]i$ increases. We also demonstrated that 10 μM of DHEA inhibited NMDA-induced NOS activity and NO production. Furthermore, L-NMMA, a 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 which plays the key role in DHEA-mediated neuroprotection. Because previous reports showed that DHEA inhibited lipopolysaccharide (LPS)-induced microglial NO production by inhibiting iNOS (Wang et al., 2001) and that DHEA activated eNOS through a specific plasma membrane receptor coupled to $G_{\alpha i2,3}$ (Liu et

al., 2002) or through mitogen-activated protein kinases (Simoncini et al., 2003), the modulatory effect of DHEA on the NOS activity remains undetermined. Further, 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 (ROS). The mitochondrial function is most important to prevent the NMDA-induced neurotoxicity, because the mitochondria plays a key role on productions of ROS (Lee et al, 2002). Further, 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 et al, 1999) and NADH dehydrogenase (Sonka et al, 1989).

Sigma receptor ligands prevent the neuronal death associated with

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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 et al., 1997). Since 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 et al., 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 VGCC, inhibition of 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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Footnotes

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Figure legends

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, **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, **p < 0.01 compared with the values elicited by the same concentration of NMDA with vehicle.

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] Pre-treatment; steroids were added 24 h before NMDA exposure. [3]

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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; □ , Pre-treatment; ▩, Co-treatment; ▨ , Post-treatment; ▤ ,Co + post-treatment; ■. Each value represents the mean of values from four independent experiments. **p < 0.01 compared with each "NMDA only".

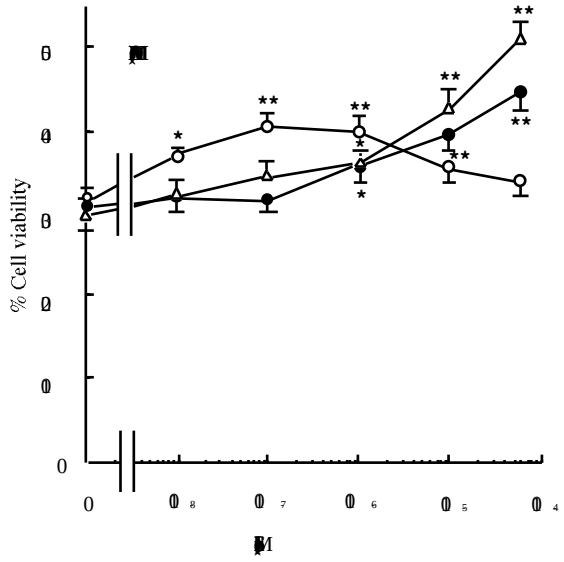
Fig. 3. Neuroprotective effect of β-estradiol through inhibition of NMDA-induced $[Ca^{2+}]i$ increase and L-type VGCCs. (A) A typical example of Ca^{2+} response to 10 μM NMDA exposure for 30 sec. Cells were perfused with steroids 5 min prior to the second application of NMDA. Bars represent periods 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, **p < 0.01 compared with the values elicited by 10 μM NMDA stimulation without steroid. (C) Neuroprotective effects of nifedipine and MK801 on NMDA-induced neurotoxicity. Ten μM nifedipine and 10 μM MK801 were added at the same time as or after exposure to 100 μM NMDA. N=4, **p < 0.01 compared with NMDA only.

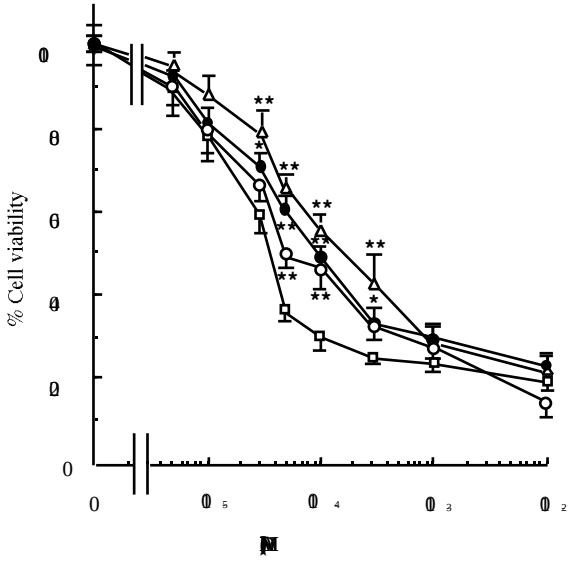
Fig. 4. Inhibition of NMDA-induced NO production by DHEA. Time courses of effects of β-estradiol, DHEA and DHEA-S on NMDA-induced NO production. Cells were exposed to 100 μM NMDA for 5, 15, 30, 60 min without steroid (vehicle; □) and in the presence of 10 μM β-estradiol (△), 10 μM DHEA (●) or 100 nM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

Fig. 6. Effects of β-estradiol, DHEA and DHEA-S on SNP-induced neurotoxicity. Cells were exposed to 1 mM SNP for 15 min. 24 h 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.


% Cell viability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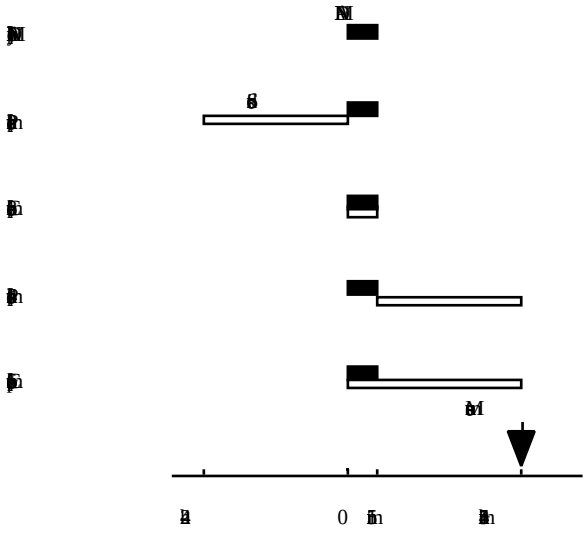

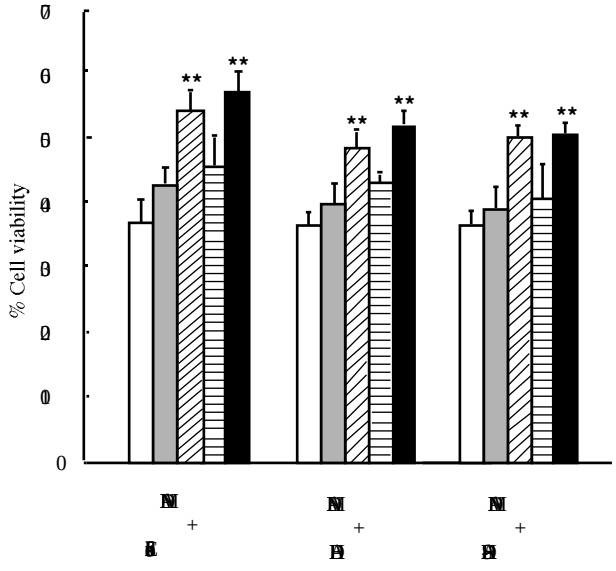
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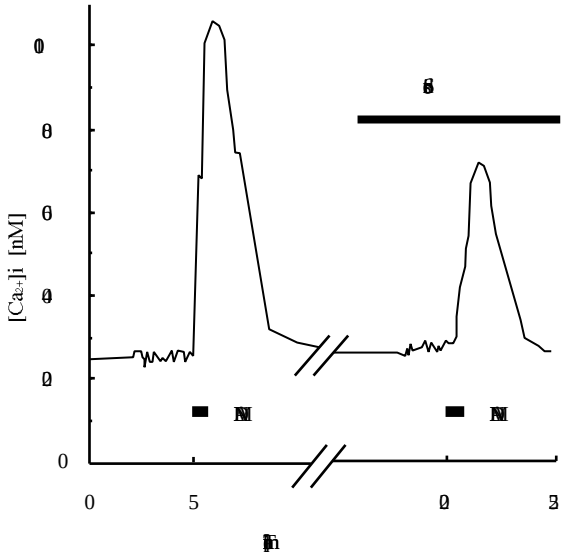
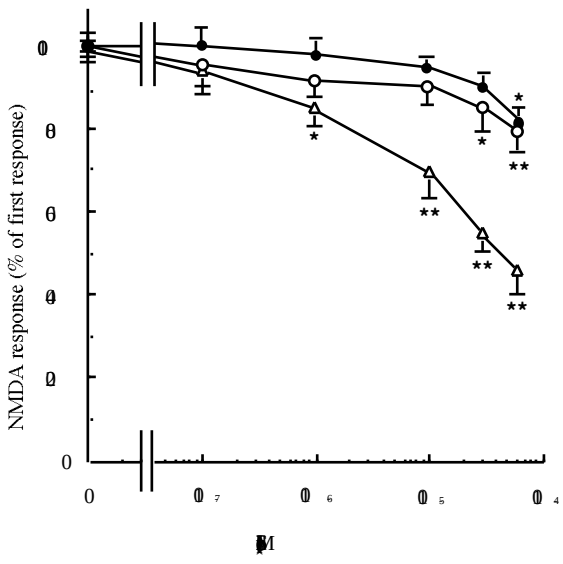

[Ca2+]i [nM]
0
5
0
3


NMDA response (% of first response)
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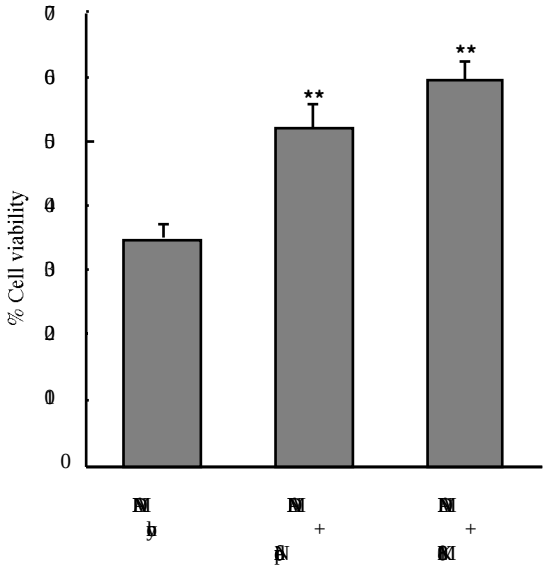
0
0
4


% Cell viability
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0
+
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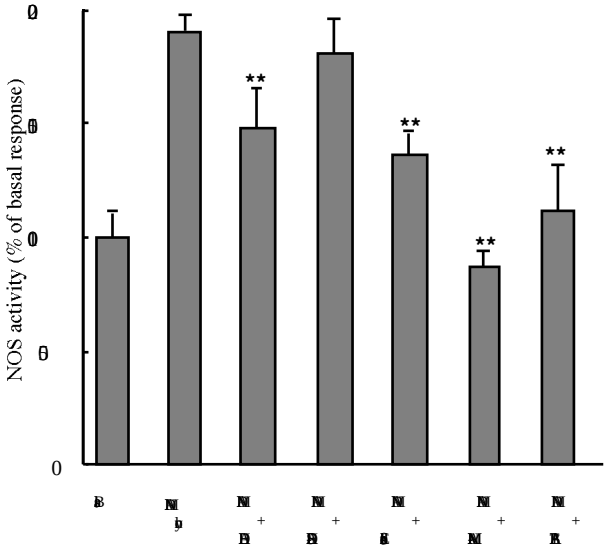
Fig.


NOS activity (% of basal response)
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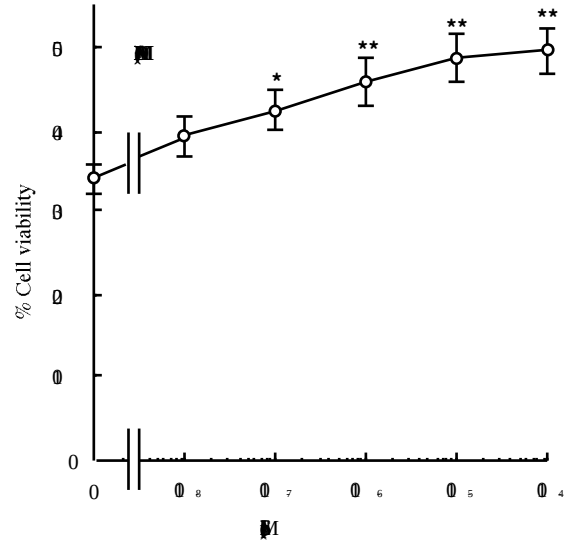
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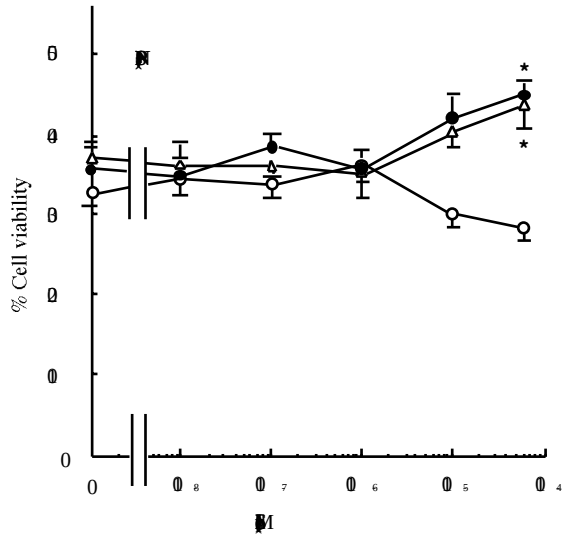
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