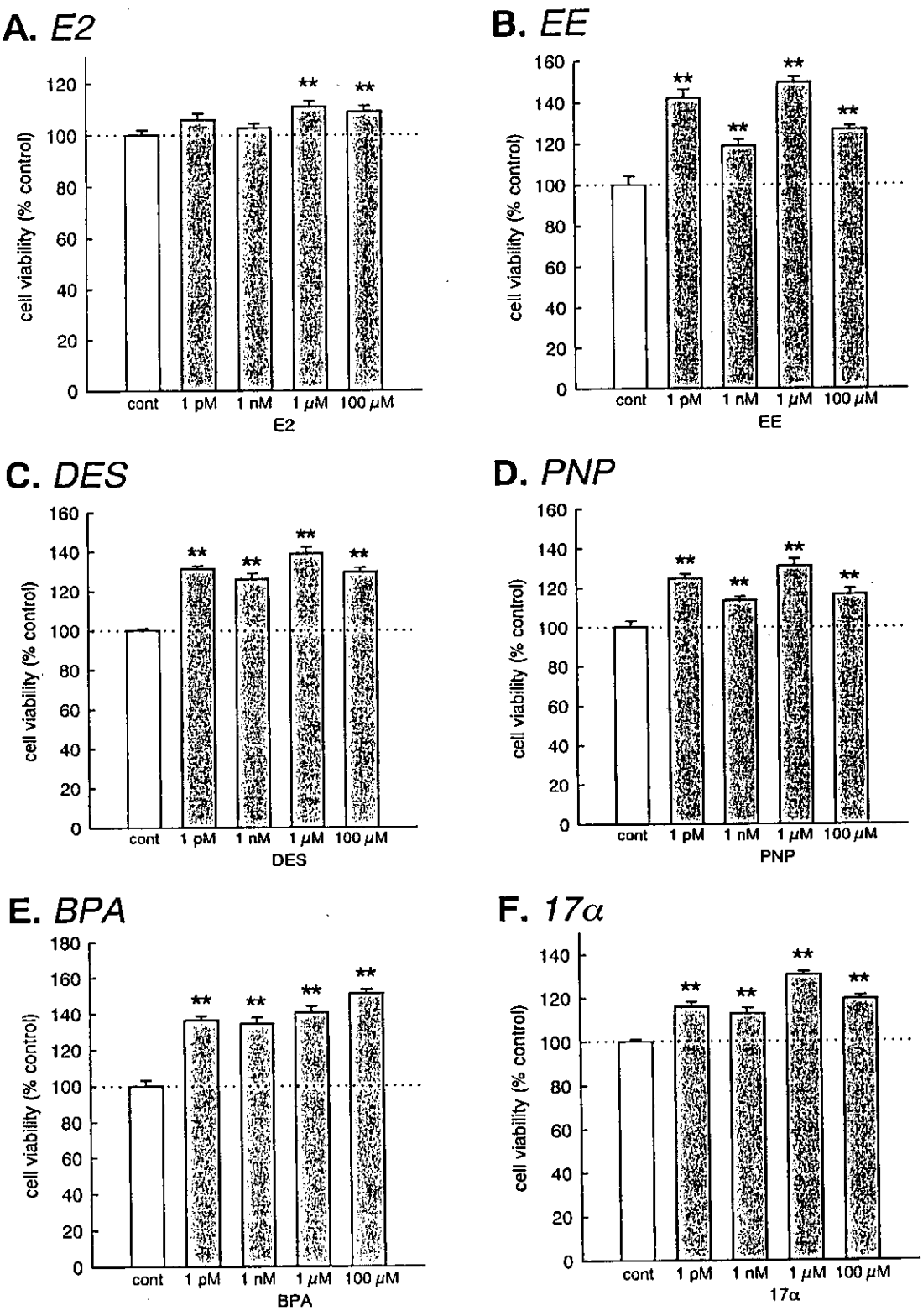

図8　培養アストロサイトの MTT reduction に対するエストロゲンおよびその類縁物質の作用

表示の濃度のE2, EE, DES, PNP, BPA, 17α で 24時間処理後，MTT reduction 測定を行った．E2 では 1, 100 μM において，その他の化合物では全ての濃度において MTT reduction の有意な上昇が観察された．（**: p<0.01 vs. control group, N=6, Dunnett's test.）

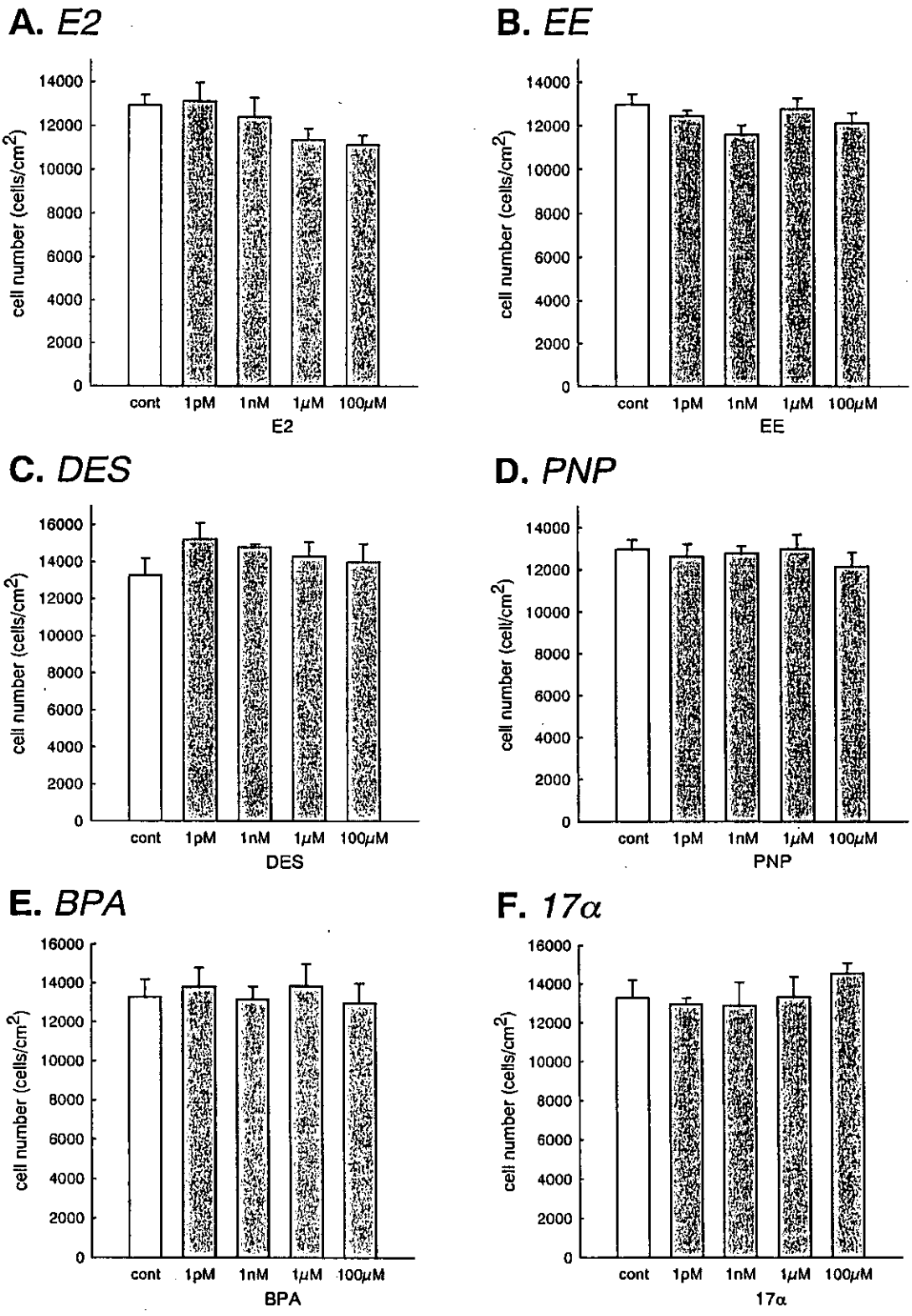

図9　培養アストロサイトの生存細胞数に対するエストロゲンおよびその類縁物質の作用

表示の濃度のE2, EE, DES, PNP, BPA, 17α で 48 時間処理後，ヘマトキシリン染色により生存細胞数を計数した．これらの全ての化合物は生存細胞数に何ら影響を与えなかった．

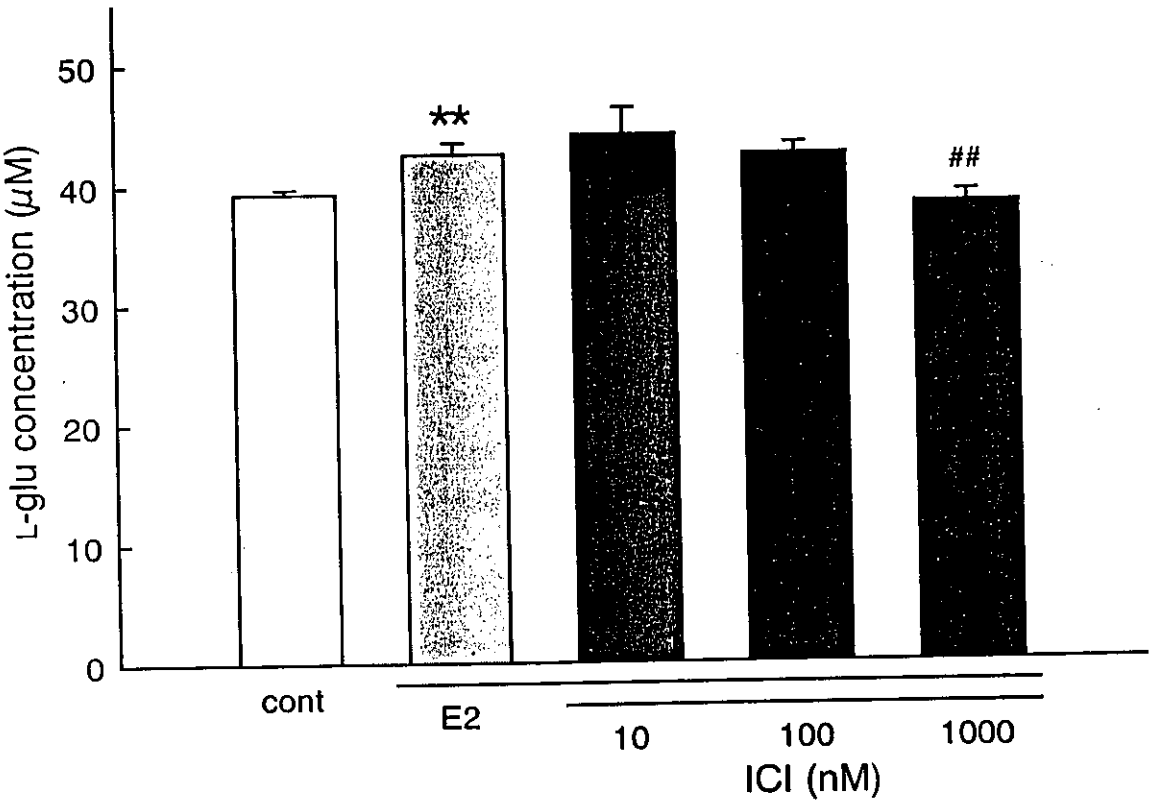

図10　17β-estradiol による L-glutamate 取り込み阻害に対する ICI 182, 780 の作用

E2 1 μM 24 時間処理によって引き起こされた L-glu 取り込み阻害は ICI との共添加により用量依存的に阻害された．(**: p<0.01 vs. control group, ##: p<0.01 vs. group exposed to E2, N=6, Duncan's test following ANOVA.)

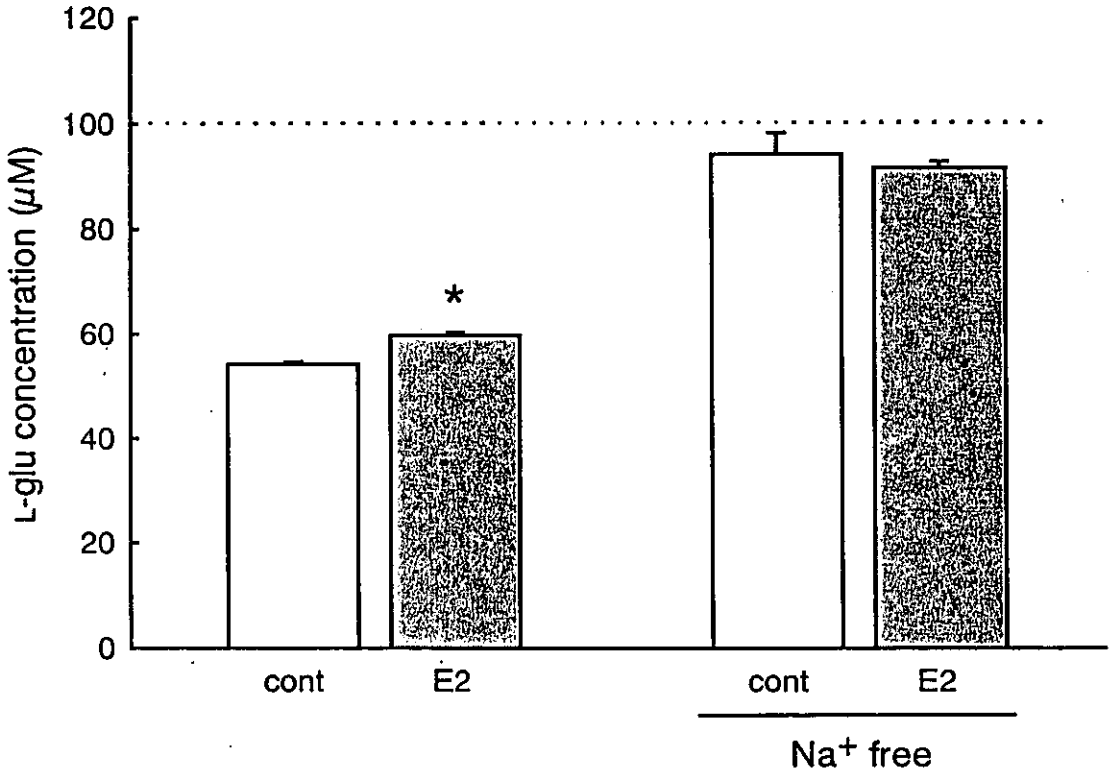

図11　17β-estradiol による L-glutamate 取り込み阻害に対する細胞外Na^+の影響

E2 100 μM で 24時間処理後 Na^+ free 条件に切り換え，100 μM の濃度でL-glu を添加し120 分後に細胞外グルタミン酸濃度を測定した．Na^+ free 条件では L-glu 取り込みがほぼ完全に抑制され，この条件ではE2 は何ら作用を示さなかった．
（*: $p<0.05$ vs. control of each group, N=4, Dunnett's test.）

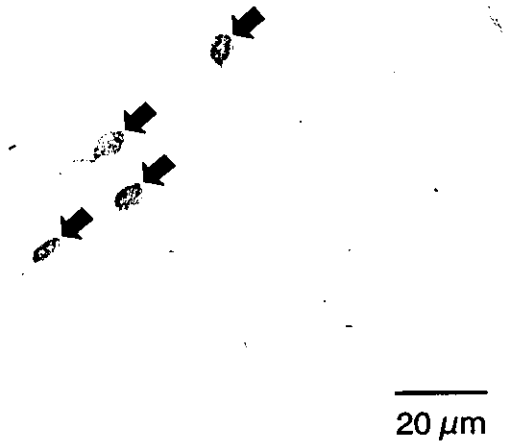

図12　BrdU 陽性アストロサイトの典型的光学顕微鏡像
再播種 3 日後に10 μM の濃度の BrdU で 1d 処理し，免疫組織化学的に BrdU 陽性細胞を染色した．また，総細胞数を計数するため，抗 BrdU 染色の後ヘマトキシリン染色を行った．矢印はBrdU 陽性細胞を示す．

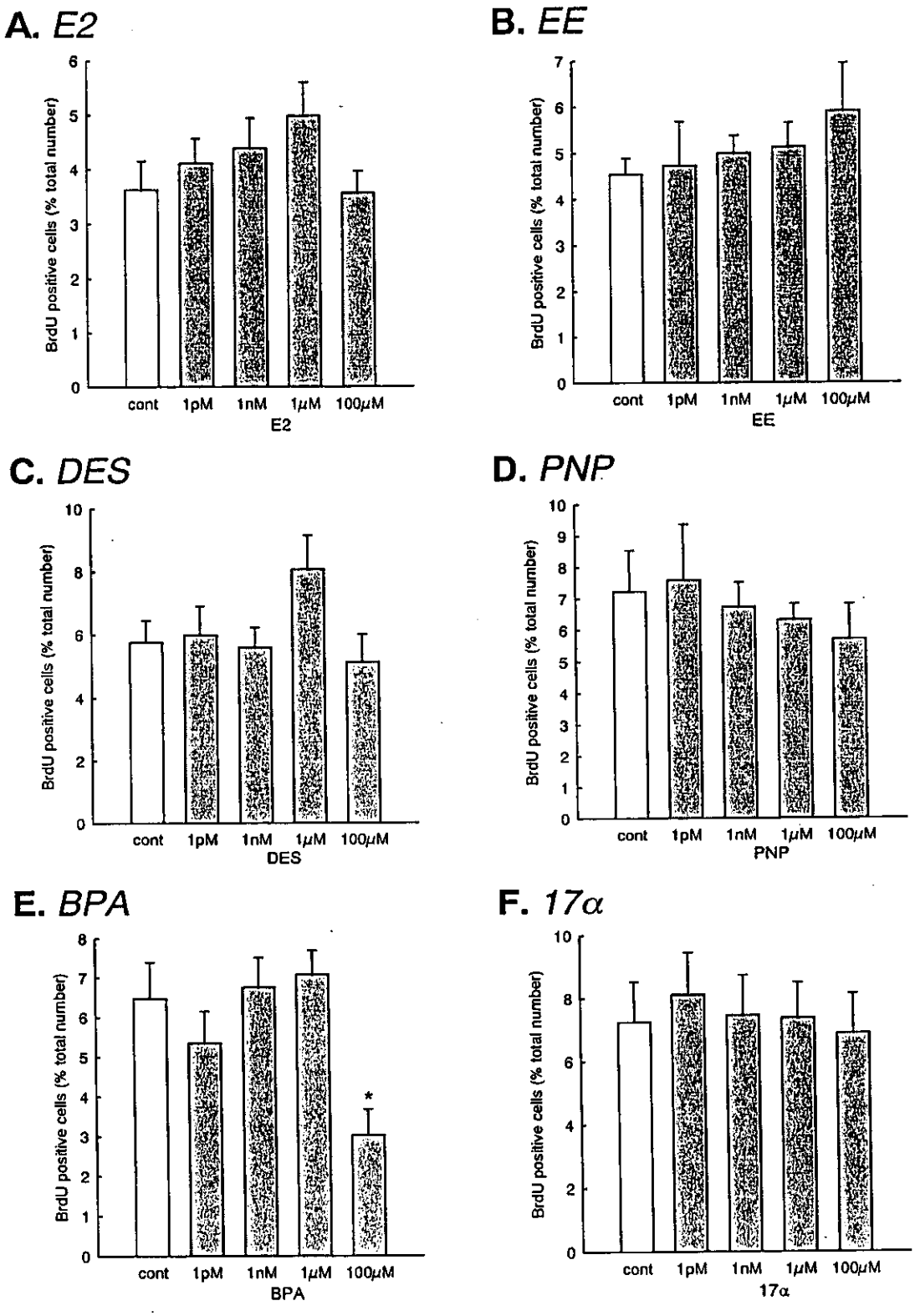

図13　培養アストロサイトの細胞分裂活性に対するエストロゲンおよびその類縁物質の作用

再播種 3 日後に表示の濃度のE2, EE, DES, PNP, BPA, 17α で 24 時間処理後，10 μM の濃度で BrdU 24 時間処理し，免疫組織化学的に BrdU 陽性細胞を染色，計数した．E2 および EE は BrdU 陽性細胞の含有率を上昇させる傾向があった．(*: $p<0.05$ vs. control of each group, N=4, Dunnett's test.)

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(資料3)

Effects of 17β-estradiol and xenoestrogens on the neuronal survival in the organotypic hippocampal culture.

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Short title: Effects of xenoestrogens on the hippocampal neurons

Key words: xenoestrogen, 17β-estradiol, bisphenol A, hippocampus, organotypic slice culture, glutamate

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Abstract

Xenoestrogens are nonsteroidal, man-made compounds that mimic the actions of estrogens through interactions with estrogen receptors (ERs). Although xenoestrogens have been received a great deal of attention as possible causes of brain disfunctions, little information concerning the effects of xenoestrogens on the central nervous system (CNS) is available. In this study, we investigated the effects of 17β-estradiol (E2) and four xenoestrogens (17α-ethynylestradiol (EE), diethylstilbestrol (DES), *p*-nonylphenol (PNP) and bisphenol A (BPA)) on the neuronal survival using organotypic hippocampal slice culture. When the cultured hippocampal slices were exposed to glutamate (1 mM, 15 min), the CA1-selective neuronal damage was induced. The pretreatment with E2 (24 hr) selectively exacerbated the CA3 neuronal damage caused by glutamate and the maximal effect of E2 was observed at 1 nM. The ERs antagonists, tamoxifen and ICI 182,780 (ICI), did not affect the effect of E2, indicating that the effect is mediated through mechanisms other than ERs. The four xenoestrogens likewise exacerbated the CA3 neuronal damage caused by glutamate and the maximal effects were observed at 1 nM. E2 and BPA (1 nM) equally increased the expression of NMDA receptor in CA3 and upregulated the spine density of the apical portion of CA3 dendrites. These compounds also enhanced the spouting of mossy fibers to CA3 neurons. These results suggest that the exposure to E2 and xenoestrogens during developmental stage results in marked influence on the synaptogensis and neuronal vulnerability through mechanisms other

than ERs.

Introduction

Xenoestrogens are nonsteroidal, man-made compounds that enter our body by ingestion or adsorption and mimic the actions of estrogens through interactions with ERs. Xenoestrogens include a number of substances such as pesticides and industrial by-products. Since it was reported that preschool children who had been exposed to pesticides demonstrated disadvantages in eye-hand coordination and 30-min memory [1], xenoestrogens have been received a great deal of attention as possible causes of brain disfunctions. However, little information concerning the effects of xenoestrogens on the CNS is available. In the CNS, both of ERα and ERβ are widely expressed and estrogens are thought to have diverse roles in regulating the structures and the functions of neuronal systems [2, 3, 4, 5]. In addition to well-known ER-mediated genomic actions, recent reports exhibit that estrogens have non-genomic effects on neuronal cells through mechanisms other than ERs [6, 7, 8]. It is unclear that xenoestrogens also have similar diverse effects on the CNS neurons.

To investigate the effects of xenoestrogens on the CNS neurons, we employed the organotypic hippocampal slice culture. In contrast to dissociated neuron cultures, organotypic slice cultures maintain neuronal configurations and region-dependent cellular properties similar to those found *in vivo* [9, 10]. The most important example of such properties is that cultured hippocampal slices reveal the selective vulnerability of CA1 neurons: CA1 neurons are selectively damaged by ischemic insults [11] and glutamate [12] in cultured hippocampal slices.

In this study, we first investigated the effects of E2 and four xenoestrogens (EE: an estrogen used for oral contraceptive pills; DES: a synthetic estrogen for preventing miscarriages; PNP: the degradation product of surface active agents used as a supplement of resins; BPA: a content of canned food, dental sealants and composites) on the neuronal survival in cultured hippocampal slices. We also investigated the effects of these compounds on the expression of NMDA receptor, spine density and mossy fiber sprouting which may affect the neuronal survival.

Materials and methods

Organotypic hippocampal culture

Organotypic cultures of hippocampi were processed using the interface method [9] according to Sato and Matsuki [12]. Brains were rapidly removed from 8-day-old Wistar rat pups, and 200 μm thick horizontal entorhino-hippocampal slices were made using a microslicer. Slices were maintained in cold Gey's balanced salt solution supplemented with 6.5 mg/ml glucose bubbled with 95% O_2 and 5% CO_2. Medial entorhino-hippocampal slices were placed on the transparent membranes (Millicell-CM, Millipore, Bedford, MA), and set in 6-well tissue culture plates containing 0.7 ml of the culture media consisting of 50% minimal essential medium, 25% Hank's balanced salt solution (HBSS), and 25% donor horse serum supplemented with 6.5 mg/ml glucose, 50 U/ml penicillin G potassium, and 100 μg/ml streptomycin sulfate. The slices were cultured at 37°C in a moist 5% CO_2 atmosphere and the culture media were changed every other day.

Drug treatment

E2 and xenoestrogens were dissolved at 10 mM in ethanol and added to culture media to yield final concentrations. The slices were incubated for 24 hour with 1 ml of the media including these compounds. Tamoxifen (sigma, St. Louis, MO) and ICI (Tocris, Ballwin, MO) were dissolved at 1 mM in ethanol and co-applied with E2 at final concentrations.

Glutamate was dissolved at 100 mM in phosphate buffered saline (PBS) and added to the media to yield final concentrations. On the 10th day *in vitro* (DIV), 1 ml of the media including glutamate was added to both above and below the membrane. The cultures were then incubated at 37°C for 15 min, washed three times with HBSS and again incubated with fresh media for 24 hr for recovery. These procedures are based on the glutamate toxicity study by Sato and Matsuki [12].

PI uptake assay

Neuronal viability was determined by PI uptake assay [12]. PI was dissolved at 500 μg/ml in PBS and added to the recovery media at 5 μg/ml. After the 24 hr-incubation with this media, the slices were washed three times with HBSS and the fluorescent images were obtained by confocal microscopy using a 4× objective.

Immunohistochemistry

Slices were washed three times with 2 ml of PBS for 5 min and fixed with 4% paraformaldehyde (PFA) (Wako Pure Chemical, Osaka, Japan) in 0.1 M phosphate buffer (PB) for 30 min. After washing with PBS, the slices were treated with 0.3% Triton X-100 in PBS for 60 min at room temperature, and blocked with PBS containing 10% Block Ace (Dainippon-Seiyaku, Osaka, Japan) at 4°C overnight. They were then incubated with rabbit polyclonal IgG to 23 residue synthetic peptide corresponding to the C-terminus of rat NR1 subunit of NMDA receptor

(Upstate Biotechnology, Lake Placid, NY) diluted at 1:100 in the vehicle (PBS containing 10% Block Ace) for 8 hr at 4°C. After wash, they were incubated with Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, Eugene, OR) diluted at 1:200 in the vehicle for 4 hr at 4°C. After washing with PBS, fluorescent images were obtained by confocal microscopy using a 4× objective or a 60× oil immersion objective.

DiI staining

Slices were washed three times with 2 ml of PBS for 5 min and fixed with 4% PFA in 0.1M PB for 30 min. The fixative above the membrane was removed and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes, Eugene, OR) crystals were embedded in CA1 and CA3 pyramidal cell layers under the microscope. After the incubation at room temperature for 3 days, the morphology of neurons were observed as fluorescent images obtained by confocal microscopy using a 60× oil immersion objective.

Confocal microscopy

Fluorescent images were visualized by μ-Radiance laser scanning confocal system (BioRad, Hercules, CA) equipped with an inverted microscope (Nikon, Tokyo, Japan). Slices were observed using a 4× objective or a 60× oil immersion objective. Data of each session were collected at the same gain and black level settings. When the 60×

objective was used, horizontal optical sections were taken at 10 μm steps and the resultant z-series were summed to a flat image.

TSQ staining

Mossy fiber terminals were visualized by staining of Zn^{2+} in the synaptic vesicles according to the modified protocol by Frederickson et al. [13]. A working solution of N-(6-methoxy-8-quinolyl)-p-toluenesulfonamide (TSQ) was prepared by dissolving 1.9 g sodium acetate and 2.9 g of sodium barbital in 100 ml of triple deionized water and then adding 0.1 ml hot ethanol that contained 1.5% TSQ. The working solution was made immediately before using and adjusted to pH 10.0. After wash with HBSS, cultured slices were fixed with 8 ml of methanol for 10 min in Petri dishes. After the treatment with 8 ml of acetone for 3 min, the slices were washed with PBS three times for 5 min and immersed in 8 ml of TSQ solution for 5 min. After wash with PBS, slices were carefully removed from the membranes and placed on clean microscope slides. TSQ florescence (385 nm) emitted by excitation at 340 nm was digitally imaged by an inverted microscope equipped with an intensified charged-coupled device camera and a digital image processor (Argus 50/CA, Hamamatsu photonics, Hamamatsu, Japan). Sixteen trial images obtained with a 4× objective were averaged to improve the signal-to-noise ratio.

Image analysis

Image analysis was performed using a graphic software (Photoshop ver. 5.5, Adobe Systems, Mountain View, CA). For measuring fluorescence intensity, five square windows (10000 μm^2 each) were placed on desired parts in the fluorescent regions of the slices and the fluorescence intensity was obtained by measuring averaged gray-scale values of selected windows. The intensities of these 5 windows were averaged and the background intensity was subtracted. In PI uptake experiments, data were normalized to 100% cell damage measured from the slices which had been exposed to TritonX-100 for 4 hours. The data of PI uptake and immuohistochemical staining were analyzed statistically for 4 slices in each group and those of TSQ staining were analyzed for 15-20 slices in each group. The numbers of spines were counted from 8-16 dendrites in CA1 and CA3, and the spine densities were expressed as the numbers of spines per 10 μm.

Statistical analysis

All the numerical data were given as means ± S.E. The data were analyzed by Dunnett's test.

Results

E2 alone (1 pM to 100 μM, treated for 24 hr) had no effect on the neuronal viability in the all regions of the cultured hippocampal slices (data not shown). However, E2 affected the neuronal vulnerability to glutamate (Fig. 1). We visualized injured or dead cells by PI, which entered cells that had lost membrane integrity and emitted fluorescence when it bound to nucleic acids. As shown in Fig. 1A (left) and B, glutamate (1 mM, 15 min) induced region-dependent neuronal damage (CA1>CA3>DG). The pretreatment with E2 (24 hr) before the exposure to glutamate markedly exacerbated the neuronal damage in CA3 at 1 nM and higher concentrations (Fig. 1A, right and B). The maximal effect was observed at 1 nM (143.0% of the group exposed to glutamate alone) followed by a slight decline at 100 μM. Although E2 also exacerbated the neuronal damage in DG at 1 pM, the effect was not observed at higher concentrations. By contrast, E2 attenuated the neuronal damage in CA1 at 1 pM (69.6% of the group exposed to glutamate alone).

To examine whether or not E2 exacerbates the CA3 neuronal damage through binding to ERs, two distinct ERs antagonists, tamoxifen (100 nM-1 μM) or ICI (10 nM-1 μM) was co-applied with E2. In preliminary experiments, we confirmed that the glutamate-induced damage was not affected by either of these antagonists (data not shown). Neither tamoxifen (Fig. 2A) nor ICI (Fig. 2B) inhibited the effect of E2, indicating that E2 exacerbated the CA3 neuronal damage through signaling pathways different from ERs.

We also investigated the effects of four xenoestrogens (EE, DES, PNP and BPA) on the neuronal vulnerability to glutamate in cultured hippocampal slices (Fig. 3). All of these compounds markedly exacerbated the CA3 neuronal damage, and revealed the maximal effects at 1 nM followed by declines at higher concentrations. The similar concentration dependence among these compounds is in contrast to their various binding affinities to ERs [14]. CA1 neuronal damage was attenuated by high concentrations of DES (100 μM), PNP (1 and 100 μM) or BPA (100 μM). DG neuronal damage was exacerbated by DES at 1 nM and attenuated by PNP at 100 μM.

To clarify the cause for the increased vulnerability to glutamate of CA3 neurons, we investigated the effects of E2 and BPA on the expression of NMDA receptor, spine density and mossy fiber sprouting. In ER-binding assays, the dissociation constants (Kd) of E2 for ERα and ERβ are 0.1 nM and 0.4 nM [15]. BPA is the weakest estrogen among the xenoestrogens used in this report and requires 300 and 2000-fold higher concentrations than E2 for binding to ERα and ERβ, respectively. Considering these findings, we compared the effects of E2 and BPA at 1 nM, a concentration at which E2 alone can activate ERs.

We examined the effects of E2 and BPA on the expression of NMDA receptor by immunostaining NR1 protein, an indispensable channel subunit of NMDA receptor. In control slices, the neuronal layers in CA1, CA3 and DG were NR1 immunopositive, and the strongest signal was observed in CA1 (Fig. 4A, left). After the exposure to E2 (1 nM, 24 hr),

the CA3 signal was profoundly increased (152% of the control group, fig. 4A, right and B). Although slight increases were also observed in CA1 and DG, these changes were weaker than that of CA3. A recent report has shown that the apical dendrites of CA3 neurons (stratum lucidum) scarcely express NR1. Thus, we confirmed the induction of NR1 by E2 on the CA3 apical dendrites by double staining with DiI (Fig. 4C, red) and NR1 immunostaining (Fig. 4C, green). Although NR1 signal was weak on the CA3 apical dendrites in the control slices (Fig. 4C, left), the patchy yellow signals were found on the CA3 apical dendrites in the E2-treated slices, suggesting that E2 promotes the expression of NR1 (Fig. 4C, right). BPA (1 nM, 24 hr) also increased the expression of NR1 in CA3 significantly (133% of the control group, Fig. 4B), but had no effect on that in CA1 and DG.

Spines were visualized by staining with DiI, a carbocyanine dye. E2 and BPA (1 nM, 24 hr) significantly increased the spine density of apical portion of CA3 dendrites (158% and 187% of the control group, respectively), whereas they had no effects on that of basal portion (Fig. 5B). Typical morphology of CA3 apical dendrites after the exposure to E2 was shown in Fig. 5A (right). These compounds had no effects on the spine densities of both portions of CA1 dendrites (data not shown).

As shown above, the increase in spine density was observed specifically in the apical dendrites of CA3 neurons. These dendrites are the postsynaptic sites of mossy fiber-CA3 synapses. Thus, we next investigated the effects of E2 and BPA on mossy fiber terminals in these