synapses. We visualized the mossy fiber terminals by staining Zn^{2+} in synaptic vesicles in mossy-fiber terminals with TSQ, a quinoline that emits strong fluorescence when it chelates to Zn^{2+}. In control slices, TSQ fluorescence was observed in the stratum lucidum of CA3 and in the hylus of DG (fig. 6A, left). When the slices were pretreated with non-fluorescent Zn^{2+} chelators, dithizone and N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), the TSQ fluorescence completely disappeared, indicating that TSQ emits fluorescence only when it binds to endogenous Zn^{2+} (data not shown). When the slices whose DG region had been dissociated on 1 DIV were stained, TSQ fluorescence in the stratum lucidum completely disappeared (data not shown), indicating that this fluorescence localizes in the mossy fiber terminals. E2 and BPA significantly increased the TSQ fluorescence in the stratum lucidum (161% and 131% of the control group, respectively; Fig. 6A and B), suggesting that these compounds enhance the sprouting of mossy fiber terminals. Interestingly, these compounds also enhanced the signal in the DG hilus.

Discussion

We investigated the effects of E2 and xenoestrogens on the CNS neurons using organotypic hippocampal culture. Our results are summarized as follows: 1) E2 selectively exacerbated the CA3 neuronal damage caused by glutamate, 2) the effect was mediated through mechanisms other than ERs, 3) xenoestrogens also selectively exacerbated the CA3 neuronal damage caused by glutamate at the same concentration as required for the exacerbation by E2 (1 nM), 4) E2 and BPA equally increased the expression of NMDA receptor in CA3, 5) E2 and BPA equally increased the spine density of apical portion of CA3 dendrites, and 6) E2 and BPA equally enhanced the spouting of mossy fiber terminals to CA3 neurons.

Little information is available concerning the effects of E2 and xenoestrogens on the CNS neurons during postnatal developmental stage. We made hippocampal slices from 8 day-old postnatal (P8) rats and cultured for 10 days with the medium containing gelding horse serum, in which the levels of estrogens were under the detection limit. It has been reported that during postnatal development, the capability of estrogen binding protein is high enough and the concentrations of serum estrogens is lowered to nonphysiological levels [16]. Thus, our results can be regarded as the acute effects of xenoestrogens on the hippocampal neurons during postnatal development if these xenoestrogens can escape from the protein binding.

E2 selectively increased the vulnerability to glutamate of CA3

neurons. To investigate whether this effect is mediated through ERs or not, we used two distinct ERs antagonists, tamoxifen and ICI. Tamoxifen is a partial agonist of ERs and mimics the agonistic effect of E2 in some tissues [17]. In contrast, it has already been reported that tamoxifen completely antagonizes E2 in the CNS neuron culture [18]. ICI is a pure antiestrogen and binds to ligand-binding domains of both of ERα and ERβ [19]. Neither of these compounds affected the exacerbation by E2 of the glutamate-induced CA3 damage, indicating that the effect of E2 was mediated though mechanisms other than ERs.

In spite that the four xenoestrogens have various binding affinities to ERs (E2 ≒ EE ≒ DES>PNP>BPA) [14], they exacerbated the glutamate-induced CA3 damage at 1 nM. Saturation ligand binding analysis has revealed that the dissociation constants (Kd) of ERα and ERβ for E2 are 0.1 nM and 0.4 nM, respectively [15]. Among the four xenoestrogens used here, BPA has the lowest binding affinities to ERs and its affinities to ERα and ERβ are 2000 times and 300 times lower than E2 [15], indicating that BPA has little interaction with ERα and ERβ at 1 nM. These data also support the idea that E2 and xenoestrogens increase the vulnerability to glutamate of CA3 neurons through pathways other than ERs.

In contrast to CA3, CA1 damage was attenuated by E2 and the xenoestrogens. A recent report has shown that a portion of excitotoxicity is caused by oxidative burden [20]. Among steroids, only estrogens are known to have the capabilities of preventing neuronal death caused by

oxidative burden [21]. The neuroprotective effects of estrogens were dependent on their basic chemical properties as hydrophobic phenolic molecules and these protections were observed at concentrations higher than 1 μM [21]. In our study, the xenoestrogens exhibited the protective effect only at concentrations higher than 1 μM. Thus, the neuroprotection of these compounds observed in CA1 may have been due to their antioxidant activities. In CA3, the region-specific exacerbation may have prevailed over the antioxidant effects of these compounds. Unlike these xenoestrogens, E2 exhibited the protective effect at 1 pM in CA1. The mechanism underlying this low concentration-limited effect is unclear.

To clarify what changes in CA3 correlated to the increased vulnerability to glutamate of CA3 neurons, we examined the effects of E2 and BPA on the expression of NMDA receptor and the spine density of CA3 neurons. These compounds equally increased the expression of NMDA receptor in CA3 and the spine density of the CA3 apical dendrites, suggesting that these changes were caused through mechanisms involving pathways other than ERs. In the ovariectomized (OVX) adult rats, E2 has been shown to affect the protein level of NMDA receptor and the spine density in CA1 [22, 23, 24]. In CA1, E2 increased NMDA receptor expression by post-transcriptional regulation [22] and upregulated the spine density via ERs in NMDA receptor-dependent manner [23]. The trigger causing the increased spine density in CA3 in our experiments may have been different from that in adult OVX rats because the change observed in our study was ER-independent. The increase of spine density in CA3,

however, may also be dependent on the increase of NMDA receptor in this region.

The increase in spine density was observed specifically in the apical portion of CA3 dendrites corresponding to the postsynaptic sites of mossy fiber-CA3 synapses. We investigated the effects of E2 and BPA on the presynaptic terminal density of mossy fibers by TSQ staining. In mossy fiber terminals, abundant Zn^{2+} is localized in the synaptic vesicles [25]. The silver-amplification method (Timm-Danscher staining) has been widely used to stain Zn^{2+} in mossy fiber terminals, but the possibility of labeling other heavy metals with this method has not been definitively excluded [13]. In the present study, we visualized Zn^{2+} with TSQ, a quinoline that forms Zn^{2+} : quinoline fluorescent chelates. Although Ca^{2+} and Mg^{2+} are also biologically relevant cations that form fluorescent complexes with TSQ [26], the binding constant of TSQ with Zn^{2+} is >1000-fold higher than that for Ca^{2+} or Mg^{2+} [27]. In preliminary experiment, we confirmed that the TSQ fluorescence completely disappeared when the slices had been pretreated with another non-fluorescent Zn^{2+} chelator, dithizone or TPEN. Thus, the TSQ signals observed in the present study represent Zn^{2+} in synaptic boutons as has been reported [13].

The pretreatment with E2 and BPA equally increased the TSQ fluorescence in the stratum lucidum, indicating that sprouting or branching of mossy fiber terminals were induced by these compounds. These data suggest that E2 and BPA enhance the synaptic reorganization of mossy

fibers with CA3 neurons through mechanisms involving pathways other than ERs. In epileptic hippocampus, selective neuronal death in CA3 [28] and aberrant sprouting of mossy fibers [29, 30, 31] has been described. The elevation of neuronal activity, as observed in epilepsy, is generally thought to result in the upregulation of spines. Thus, the upregulation of NMDA receptor and spine density and the mossy fiber sprouting induced by E2 and the xenoestrogens in this study may contribute as key steps to the increased vulnerability of CA3 neurons induced by these compounds.

Mossy fibers also establish synaptic contacts with polymorphic neurons in the DG hilus. Thus, the TSQ fluorescence observed in DG may represent a small population of recurrent axon collaterals branching from parent mossy fibers. Interestingly, E2 and BPA increased the TSQ fluorescence in the hilus as well as that in the stratum lucidum of CA3. These results suggest that E2 and BPA enhance the mossy fiber reorganization, which itself is not related to vulnerability to glutamate. The trigger that caused the postsynaptic changes observed in CA3 is unclear. The increase of spine density was observed specifically in the apical dendrites of CA3 neurons. Multiple events are believed to be necessary for the synapse formation. It has been reported that afferent inputs strongly influence the shape and number of dendritic spines [32, 33, 34]. The postsynaptic changes observed in the present study might have resulted from the presynaptic reorganization of mossy fiber terminals described above.

A series of the responses observed in our study appeared to involve

mechanisms other than ERs. Recent studies have provided a large body of evidence that estrogens interact with plasma membrane binding sites/receptors, which are hypothesized to be a G-protein-coupled type and reveal the effects via cAMP/protein kinase A (PKA) signaling pathway [35]. Phosphorylation of cAMP response element binding protein (CREB) leads to the generation of new dendritic spines of cultured hippocampal neurons [36] and the neurite growth of midbrain dopaminergic neurons [37]. The responses in the present study may also be mediated through membrane binding sites/receptors coupled to intracellular transduction pathways.

Our results have raised the possibility that the exposure to E2 and xenoestrogens during the developmental stage results in marked influence on the generation of neuronal circuitry and vulnerability through unknown mechanisms other than ERs. Although the influence can occur at very low concentrations (1 nM), the risk for the nervous systems of human and other animals cannot be elucidated at present. The risk assessment and, if the risk is present, the development of procedures to avoid the influence may be necessary.

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Figure legends

Fig. 1. The effect of E2 on the glutamate-induced neuronal damage in the cultured hippocampal slice.

A. Typical PI fluorescent images of the slice exposed to glutamate alone (1 mM, 15 min) (left) and the slice pretreated with E2 (1 nM, 24 hr) before the exposure to glutamate (right).

B. Normalized PI fluorescence intensities in CA1, CA3 and DG. E2 selectively exacerbated the CA3 neuronal damage caused by glutamate and the most pronounced effect was observed at 1 nM (*: $p<0.05$, **: $p<0.01$ vs. the group exposed to glutamate alone. N=4, Dunnett's test).

Fig. 2. The effects of tamoxifen (A) and ICI (B) on the E2-induced exacerbation of the CA3 damage by glutamate. These ERs antagonists did not affect the E2-induced exacerbation (**: $p<0.01$ vs. the group exposed to glutamate alone. N=4, Dunnett's test).

Fig. 3. The effects of EE (A), DES (B), PNP (C) and BPA (D) on the glutamate-induced neuronal damage in cultured hippocampal slices. Normalized PI fluorescence intensities in CA1, CA3 and DG are shown. All of these xenoestrogens selectively exacerbated the CA3 neuronal damage caused by glutamate and the most pronounced effect was observed at 1 nM (*: $p<0.05$, **: $p<0.01$ vs. the group exposed to glutamate alone. N=4,

Dunnett's test).

Fig. 4. The effects of E2 and BPA (1 nM, 24 hr) on the expression of NR1 subunit of NMDA receptor.

A. Typical NR1 immunofluorescence in the control slice (left) and the slice treated with E2 (right).

B. Normalized fluorescence intensity in CA3. E2 and BPA significantly increased the expression of NR1 (*: $p<0.05$, **: $p<0.01$ vs. control group. N=4, Dunnett's test).

C. Typical fluorescent images of CA3 apical dendrites of the control slice (left) and the slice treated with E2 (right), double-stained with DiI (red) and NR1 immunostaining (green). The induction of NR1 by E2 on the CA3 apical dendrites was identified as yellow patches.

Fig. 5. The effects of E2 and BPA (1 nM, 24 hr) on the spine density.

A. Typical fluorescent images of DiI-stained CA3 apical dendrites of the control slice (left) and the slice treated with E2 (right).

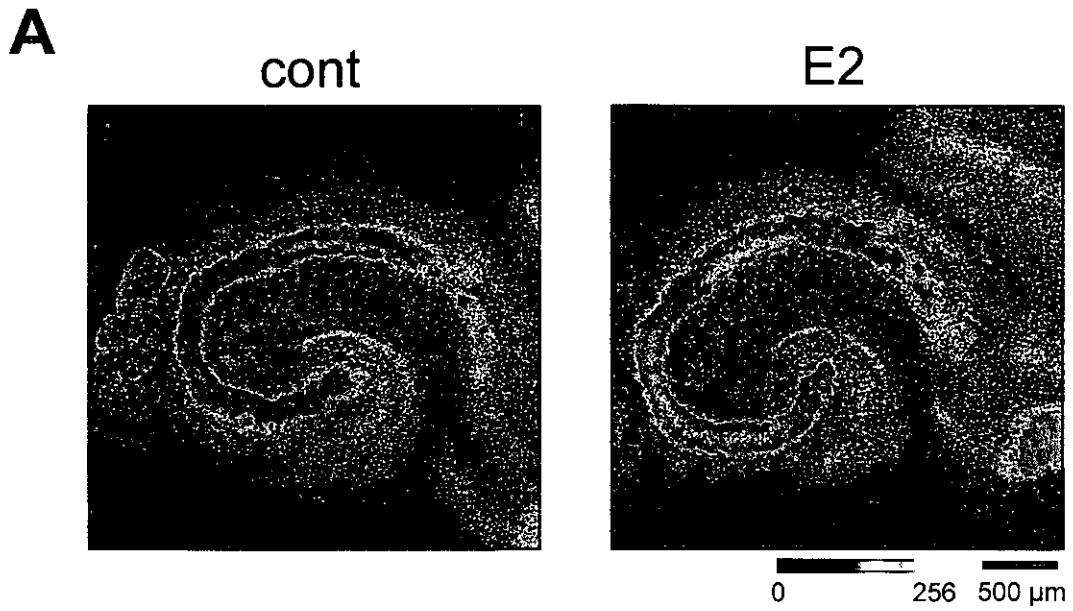
B. Spine density of the apical and basal dendrites of CA3 neurons. E2 increased the spine density of the apical dendrites (**: $p<0.01$ vs. control group. N=8-16, Dunnett's test).

Fig. 6. The effects of E2 and BPA on the mossy fiber sprouting.

A. Typical TSQ fluorescence in the control slice (left) and the slice

treated with E2 (right).

B. Normalized TSQ fluorescence intensity in the stratum lucidum of CA3. E2 and BPA significantly enhanced the mossy fiber sprouting (*: $p<0.05$, **: $p<0.01$ vs. control group. N=16-20, Dunnett's test).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256
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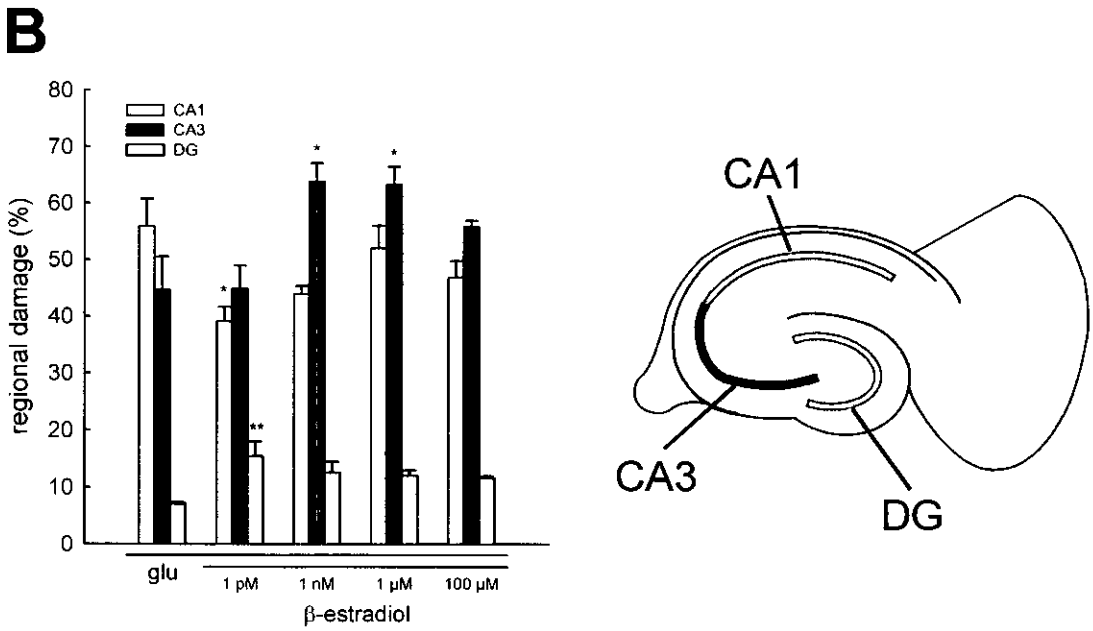
B
CA1
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DG
regional damage (%)
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10
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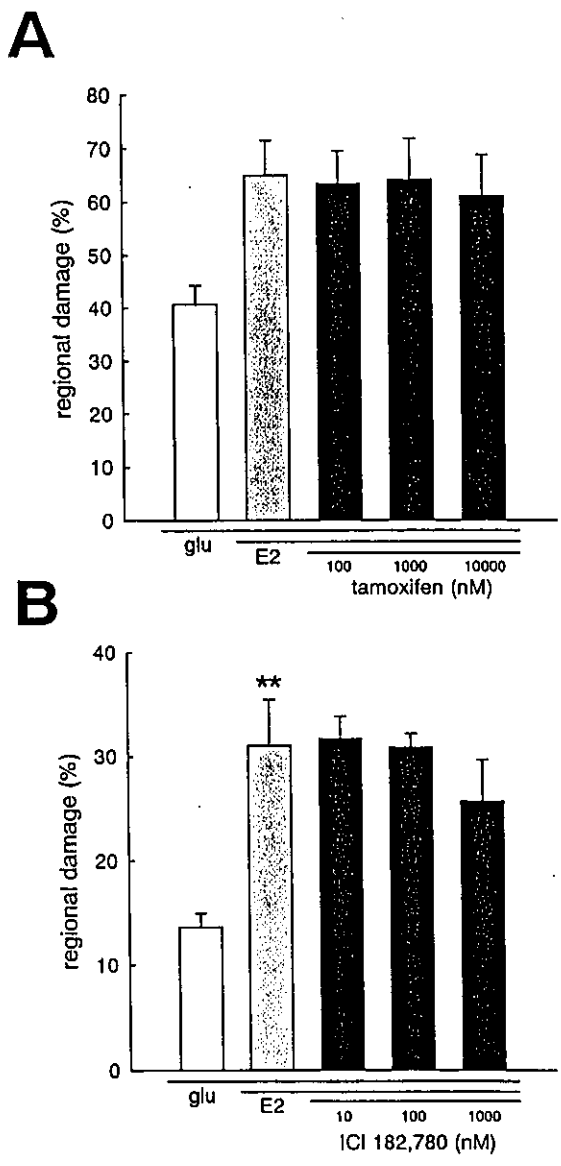
glu
1 pM
1 nM
1 µM
100 µM
β-estradiol
CA1
CA3
DG

75

Fig. 1


A
80
70
60
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30
20
10
0
regional damage (%)
glu
E2
100
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tamoxifen (nM)
B
40
30
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10
0
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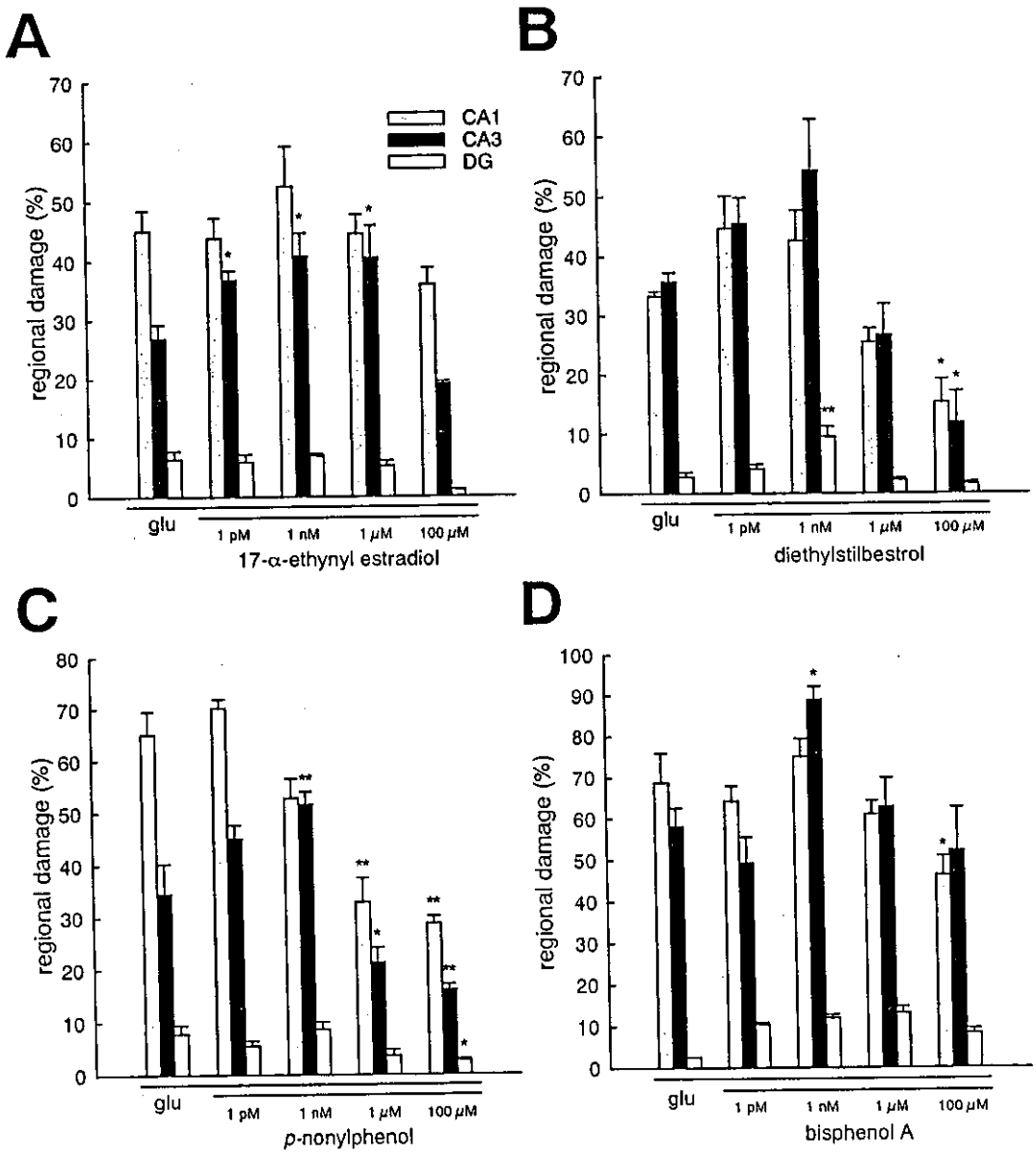
regional damage (%)
glu
E2
10
100
1000
ICI 182,780 (nM)

Fig. 2


A
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C
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CA1
CA3
DG
regional damage (%)
glu
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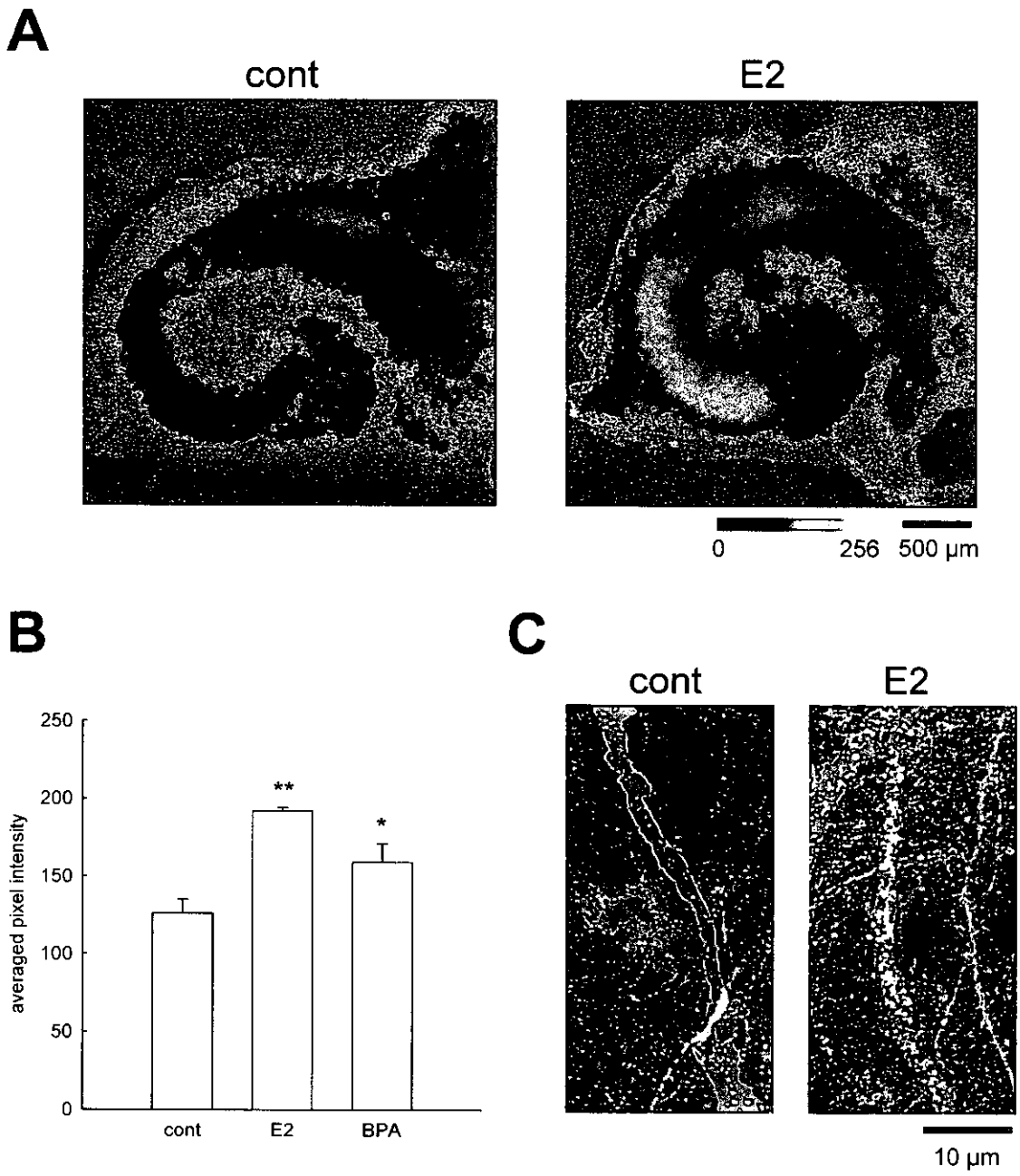
diethylstilbestrol
p-nonylphenol
bisphenol A

Fig. 8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E2
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Fig. 4