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Estrogen Enhances Depolarization-Induced Glutamate Release through Activation of Phosphatidylinositol 3-Kinase and Mitogen-Activated Protein Kinase in Cultured Hippocampal Neurons

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Changes in synaptic efficacy are considered necessary for learning and memory. Recently, it has been suggested that estrogen controls synaptic function in the central nervous system. However, it is unclear how estrogen regulates synaptic function in central nervous system neurons. We found that estrogen potentiated presynaptic function in cultured hippocampal neurons. Chronic treatment with estradiol (1 or 10 nM) for 24 h significantly increased a high potassium-induced glutamate release. The estrogen-potentiated glutamate release required the activation of both phosphatidylinositol 3-kinase and MAPK.

The high potassium-evoked release with or without estradiol pretreatment was blocked by tetanus neurotoxin, which is an inhibitor of exocytosis. In addition, the reduction in intensity of FM1-43 fluorescence, which labeled presynaptic vesicles, was enhanced by estradiol, suggesting that estradiol potentiated the exocytotic mechanism. Furthermore, protein levels of synaptophysin, syntaxin, and synaptotagmin (synaptic proteins, respectively) were up-regulated by estradiol. We confirmed that the up-regulation of synaptophysin was blocked by the MAPK pathway inhibitor, U0126. These results suggested that estrogen enhanced presynaptic function through the up-regulated exocytotic system. In this study, we propose that estrogen reinforced excitatory synaptic transmission via potentiated-glutamate release from presynaptic sites. (*Molecular Endocrinology* 17: 831–844, 2003)

THE OVARIAN STEROIDS, estrogen and progesterone, are known to influence the reproductive behavior and sexual differentiation of the brain. Moreover, substantial evidence has shown that steroids have a neuroprotective effect against disease and injury of the nervous system. For example, Chen *et al.* (1) demonstrated a protective effect of 17β-estradiol (estradiol) on CA1 hippocampal cells after ischemia in gerbils. Progesterone was also effective in preventing neural damage such as cerebral edema after traumatic brain injury (2) and ischemia (3).

Recently, it was reported that these steroids have a novel physiological role in the nervous system; estrogen contributes to synaptic function (4–12), which is believed necessary for emotional behavior or learning and memory (13–20). However, the characterization of estrogen-mediated synaptic plasticity has been limited in scope. Foy *et al.* (21) reported that treatment with estradiol rapidly ($<$10 min) enhanced *N*-methyl-D-aspartate (NMDA) glutamate receptor-mediated excitatory postsynaptic potential and hippocampal long-term potentiation. In the hippocampus CA1 region of ovariectomized rats, chronic treatment with estradiol increased the dendritic spine density (22) and the number of NMDA receptor binding sites (23, 24). These results indicate the possibility that estrogen was involved in neuronal plasticity through the potentiation of postsynaptic function. However, not only postsynaptic function but also the presynaptic system is important for neuronal plasticity. Zakharenko *et al.* (25) showed that short-term and long-term synaptic plasticity at the CA3-CA1 synapse in acute hippocampal slices was likely to depend, at least in part, on enhanced transmitter release from the presynaptic neurons. That is, there is the possibility that regulation of

Abbreviations: CBB, Coomassie brilliant blue; ER, estrogen receptor; 17β-estradiol, estradiol; GABA, γ-aminobutyric acid; HK^+, high potassium; KRH, HEPES-buffered Krebs Ringer solution; MAP2, microtubule-associated protein 2; MEK, MAPK kinase; NMDA, *N*-methyl-D-aspartate; PI 3-kinase, phosphatidylinositol 3-kinase; SDS, sodium dodecyl sulfate; TeNT, tetanus neurotoxin; T-TBS, Tween 20/Tris-buffered saline.

presynaptic function is critical for the change in synaptic transmission. However, the contribution of estrogen to the presynaptic function is not fully understood, and the signaling mechanisms underlying the estrogenic regulation of synaptic transmission are not known.

In the present study, we investigated the possibility that chronic treatment with estradiol potentiates presynaptic function. An increase in the amount of glutamate and γ-aminobutyric acid (GABA) released is expected to influence synaptic transmission because glutamate (an excitatory amino acid neurotransmitter) and GABA (an inhibitory neurotransmitter) have crucial roles in neuronal plasticity (15, 26). We found that estradiol potentiated a depolarization-induced release of glutamate through the up-regulation of exocytotic machinery. On the other hand, it had no effect on the release of GABA induced by high potassium (HK^+)-evoked depolarization. The potentiated release by estradiol was dependent on the activation of phosphatidylinositol 3-kinase (PI 3-kinase) and MAPK. We propose that estrogen enhances excitatory synaptic transmission through the increase in release of neurotransmitter, glutamate.

RESULTS

β-Estradiol Potentiated the Glutamate Release Induced by HK^+ in Cultured Hippocampal Neurons

To study the possibility that chronic treatment with estradiol potentiates neurotransmitter release, we quantified the amount of glutamate and GABA released by HK^+-induced depolarization with or without estradiol treatment. Pretreatment with estradiol (10 nM) for 24 h caused a marked increase in the amount of glutamate released by HK^+ stimulation, but basal release was not affected (Fig. 1A). On the other hand, estradiol did not have an effect on the release of GABA (Fig. 1B). α-Estradiol (a stereoisomer; 10 nM, 24 h) did not potentiate the HK^+-induced glutamate release [unaffected basal release, 53.5 ± 7.9; unaffected HK^+-evoked release, 72 ± 3.9; α-estradiol-treated basal release, 42.7 ± 15; α-estradiol-treated HK^+-evoked release, 65.1 ± 17 (nmol/well, $P > 0.1$, unaffected HK^+-evoked *vs.* α-estradiol-treated HK^+-evoked)]. In our system, the number of cultured neurons did not change with or without estradiol treatment for 24 h (Fig. 1C). Thus, estradiol did not contribute to the survival-promoting effect in this system. Next, it was confirmed that the release of glutamate was enhanced by estradiol using more mature neurons [14 d *in vitro*: unaffected basal release, 41.2 ± 10; unaffected HK^+-evoked release, 112 ± 15; estradiol-treated basal release, 40.3 ± 8.8; estradiol-treated HK^+-evoked release, 170 ± 13 (nmol/well, $P < 0.01$, unaffected HK^+-evoked *vs.* estradiol-treated HK^+-evoked)]. These results indicate that estradiol enhances the release of glutamate in mature hippocampal neurons.

To further investigate the estradiol-potentiated release, the dose-dependent effect of estradiol on the release of glutamate evoked by HK^+ was examined (Fig. 1D). Estradiol at 1 and 10 nM induced a significant increase in glutamate release compared with untreated cells (1.63- and 1.63-fold, respectively), although estradiol at 100 nM did not. Therefore, all other experiments were performed at 1 or 10 nM.

Estrogen Receptor (ER), PI 3-Kinase, and MAPK Were Involved in the β-Estradiol-Enhanced Glutamate Release

Estrogen is known to act via the ER. Thus, we tested whether the action of estradiol on the release of glutamate required ER in our system. ICI182,780 (1 or 10 μM), which is an ER antagonist, blocked the glutamate release potentiated by estradiol, and this antagonist alone had no effect (Fig. 2). It was confirmed that tamoxifen (1 μM), which antagonizes ER-mediated cellular event, also blocked this effect [unaffected basal release and HK^+-evoked, 63.7 ± 18 and 130 ± 4.8, respectively; estradiol-treated basal and HK^+-evoked, 60.7 ± 18 and 167 ± 15; tamoxifen alone, 86.0 ± 18 and 126 ± 54; tamoxifen plus estradiol, 59.2 ± 7.2 and 106 ± 29 (nmol/well, $P < 0.01$, estradiol-treated HK^+-evoked *vs.* tamoxifen plus estradiol-treated HK^+-evoked)]. These results suggest that exogenous estradiol exerts this effect through the ER.

What intracellular signaling was necessary for the action of estradiol in the regulation of synaptic function? It has been reported that estradiol protects central nervous system neurons from cell death via activation of MAPK or PI 3-kinase (27–31). Thus, we examined whether estradiol activates the MAPK pathway and/or PI 3-kinase pathway in our system. As shown in Fig. 3A, estradiol caused the phosphorylation of p44 MAPK within 5 min, and this phosphorylation mostly returned to basal level after 3 h. It was observed that this activation of MAPK was maintained until 8 h after estradiol application in some cases. p42 MAPK was also activated, although not significantly, as shown in Fig. 3A ($P = 0.09$). These phosphorylations of both p44 and p42 MAPK at 5 min were inhibited by U0126 [which inhibited MAPK kinase (MEK), an upstream of MAPK] or ICI182,780 (b in Fig. 3A). These results indicate that estradiol activates MAPK signaling through the ER.

The activation of Akt (which is downstream of PI 3-kinase) was caused by estradiol treatment within 30 min, and this was continued for 24 h (Fig. 3B). It was confirmed that the phosphorylation of Akt was inhibited by LY294002 (a PI 3-kinase inhibitor) or ICI182,780 (b in Fig. 3B), indicating that estradiol activated not only MAPK but also the PI 3-kinase pathway, and these activations occurred through ER.

Next, we tested the effect of U0126 or LY294002 on the release of glutamate potentiated by estradiol.

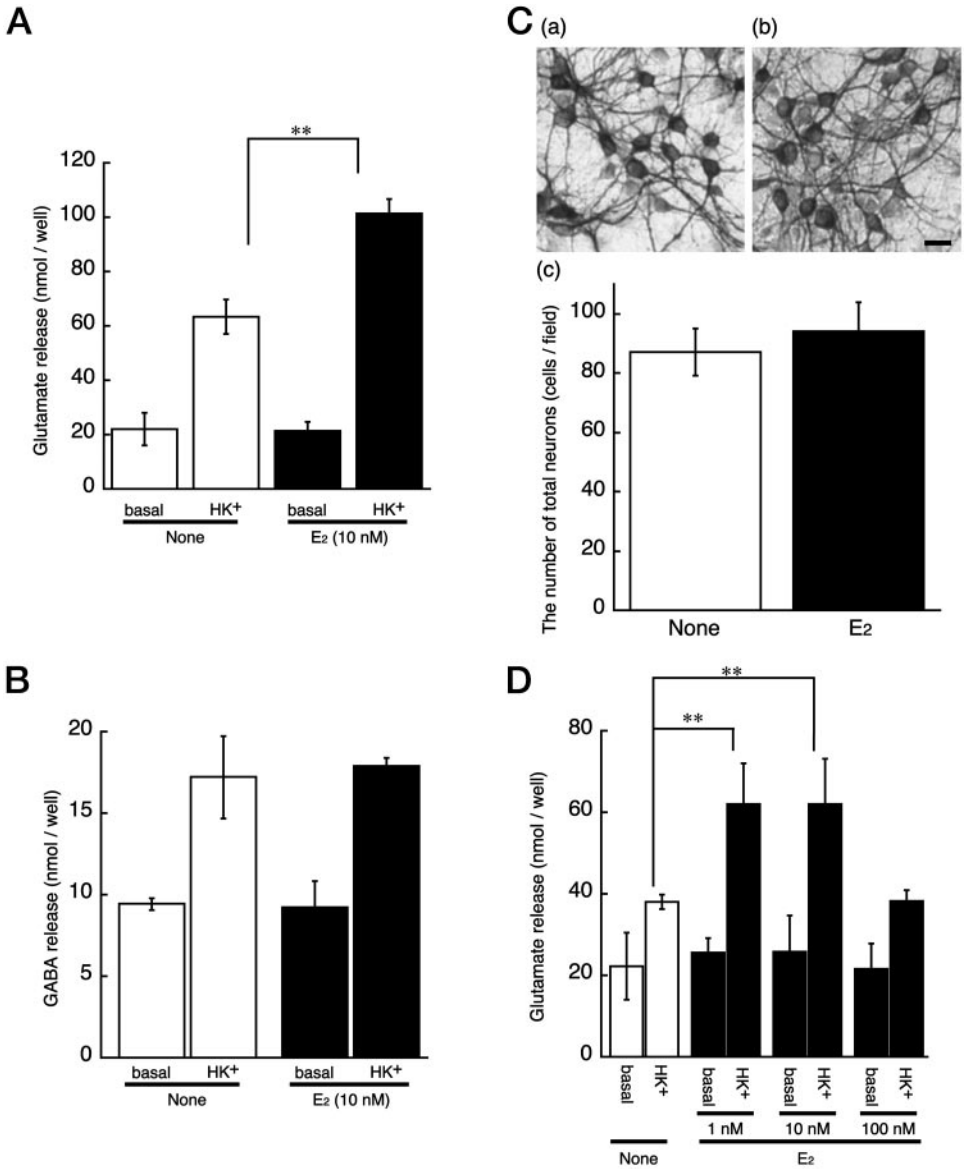

Fig. 1. Chronic Treatment with Estradiol Potentiated the HK^+-Evoked Glutamate Release in Cultured Hippocampal Neurons

Cultured hippocampal neurons were prepared from 2- to 3-d-old rats and were maintained for 8 d *in vitro* (DIV8) before the assay. Estradiol was applied to cultures from DIV7–DIV8. Then, basal and depolarization-evoked amino acid release was measured by HPLC as described in *Materials and Methods*. A, Estradiol (10 nM, 24 h) increased the release of glutamate induced by HK^+ (50 mM). Estradiol did not affect the basal release. Data represent the mean ± SD (n = 4). **, $P < 0.01$ *vs.* unaffected HK^+-evoked release (Student's *t* test). E2, β-Estradiol. B, The HK^+-induced GABA release was not affected by estradiol (10 nM, 24 h). Data represent the mean ± SD (n = 4). C, Estradiol had no effect on the survival of cultured hippocampal neurons. Immunocytochemistry with MAP2 antibody was performed. a, Untreated neurons; b, estradiol-treated neurons. Estradiol was applied at 10 nM for 24 h. *Scale bar*, 20 μm. c, The number of immunopositive cells with anti-MAP2 antibody. Estradiol did not have an effect on the number of MAP2-positive cells. Data represent the mean ± SD (n = 10). D, Hippocampal neurons were treated with estradiol for 24 h at 1, 10, and 100 nM. Treatment with estradiol at 1 and 10 nM significantly potentiated the HK^+-evoked glutamate release, whereas potentiation was not observed at 100 nM. Cells were cultured for 7 d *in vitro*. Data represent the mean ± SD (n = 3 or 4). **, $P < 0.01$ *vs.* unaffected HK^+-evoked release (Student's *t* test).

U0126 (0.1, 1, and 10 μM) blocked the estradiol-enhanced glutamate release (Fig. 4A). On the other hand, GABA release by HK^+ stimulation was not influenced by U0126 (Fig. 4B). LY294002 also blocked the estradiol-enhanced release of glutamate in a dose-dependent manner (Fig. 5A). Interestingly, the HK^+-evoked GABA release was inhibited by LY294002 in a dose-dependent manner, although potentiation in release of GABA by estradiol was not observed (Fig. 5B), implying that PI 3-kinase signaling was important for the release system of GABA. These results indicate that activation of both the MAPK pathway and PI 3-

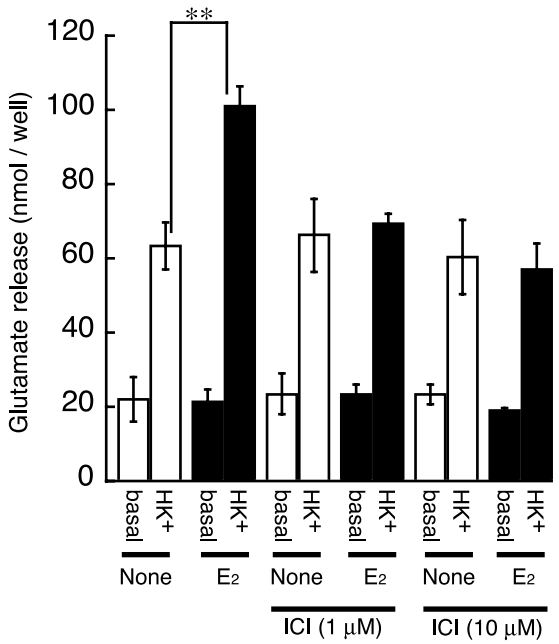

Fig. 2. Activation of the ER Was Required for Estradiol-Enhanced Glutamate Release

ER antagonist, ICI182,780 (1 or 10 μM), was added 2 h before to the application of estradiol (10 nM for 24 h). This antagonist inhibited the effect of estradiol, suggesting that estradiol exerted its effect via activation of the ER. Cells were cultured for 8 d *in vitro* before assay. Estradiol was applied to cultured neurons from DIV7–DIV8. Data represent the mean ± SD (n = 3 or 4). **, $P < 0.01$ *vs.* untreated HK^+-evoked release (Student's *t* test). E2, β-Estradiol; ICI, ICI182,780.

kinase is necessary for the estradiol-potentiated glutamate release.

β-Estradiol Increased the Loss of FM1-43 Fluorescent Intensity Induced by Depolarization

To clarify the mechanism of estradiol-potentiated release of glutamate, we performed immunocytochemical staining with antisynaptophysin antibody and imaging analysis with FM1-43 (Molecular Probes, Inc., Eugene, OR) dye. First, to determine whether the number of presynaptic sites was increased after estradiol treatment, immunocytochemical staining with antisynaptophysin antibody was performed. As shown in Fig. 6A, the number of presynaptic site (boutons like synaptophysin-immunopositive signals) was not changed by estradiol. The HK^+-evoked neurotransmitter release mostly occurred through the exocytotic system (32–37). Thus, next, to test whether estradiol activated the exocytotic system, we performed an exocytosis assay with the style dye FM1-43 (Fig. 6, B and C). FM1-43 dye is known to be uptaken into active presynaptic sites during endocytosis occurring after exocytosis triggered by HK^+ stimulation. After FM dye loading with HK^+-evoked depolarization, we analyzed the number of FM1-43-positive boutons in none-treated and estradiol-treated neurons (Fig. 6B) and found that the number of FM boutons was not changed. Then, we monitored the loss of FM intensity induced by secondary HK^+ stimulation after FM dye loading. Although FM fluorescent intensity in boutons was decreased by HK^+ stimulation both in estradiol-treated and none-treated neurons, the loss of FM fluorescence in estradiol-treated cells was significantly more than in none-treated cells (Fig. 6C). These results suggest that estradiol does not change the number of presynaptic sites, but activates the exocytotic machinery at individual presynaptic sites.

β-Estradiol Up-Regulated the Expression of Synaptic Proteins Synaptophysin, Synaptotagmin, and Syntaxin

In addition to the imaging assay with FM dye, we confirmed that the estradiol-potentiated glutamate release did not occur with tetanus neurotoxin (TeNT) treatment (Fig. 7A). TeNT was a potent inhibitor of exocytotic neurotransmitter release (38). Furthermore, under the conditions in which the extracellular Ca^{2+} influx was blocked (in the presence of nifedipine), estradiol-potentiated release was not observed [basal and HK^+-evoked release in the presence of nifedipine, 52.0 ± 8.0 and 63.0 ± 11 (none-treated); 47.1 ± 18 and 47.5 ± 10 (estradiol-treated); nmol/well, $P > 0.1$, nifedipine plus estradiol-treated basal *vs.* nifedipine plus estradiol-treated HK^+-evoked]. The results from these pharmacological experiments also indicate the possibility that up-regulation of the exocytotic system was involved. Therefore, we focused on the expression of synaptic protein, which is important for vesicle fusion in exocytosis. The change in expression of synaptophysin with or without estradiol was analyzed by Western blotting, and the levels of proteins were quantified by densitometry. As shown in Fig. 7B, treatment with estradiol for 24 h increased the expression of synaptophysin, a synaptic vesicle membrane protein. Class III β-tubulin (TUJ1) level was not changed by estradiol (Fig. 7B). The up-regulation of synaptophysin was blocked by the ER antagonist, ICI182,780, indicating that activation of the ER was involved. U0126 also blocked the increase in the expression of synaptophysin as expected. LY294002 (a PI 3-kinase inhibitor) also inhibited the up-regulation, although its inhibition was partial ($P = 0.14$). These results indicate that the up-regulation of synaptophysin expression by estradiol requires the activation of MAPK and partially requires the activation of PI 3-kinase. Next, the time-course analysis of synaptophysin expression increased by estradiol was performed. As shown in Fig. 7C, the up-regulation significantly occurred after 18-h exposure of estradiol. Furthermore, the expression of other synaptic proteins was examined. Synaptotagmin and syntaxin are known to be direct modulators of exocytosis in neurons (39). As shown in Fig. 7D, the expression of synaptotagmin and syntaxin were also increased by long-term estradiol treatment (24 h), although the levels of synapsin I, which is known to localize on synaptic vesicle membranes, were not changed by estradiol. These results suggest that estradiol enhances

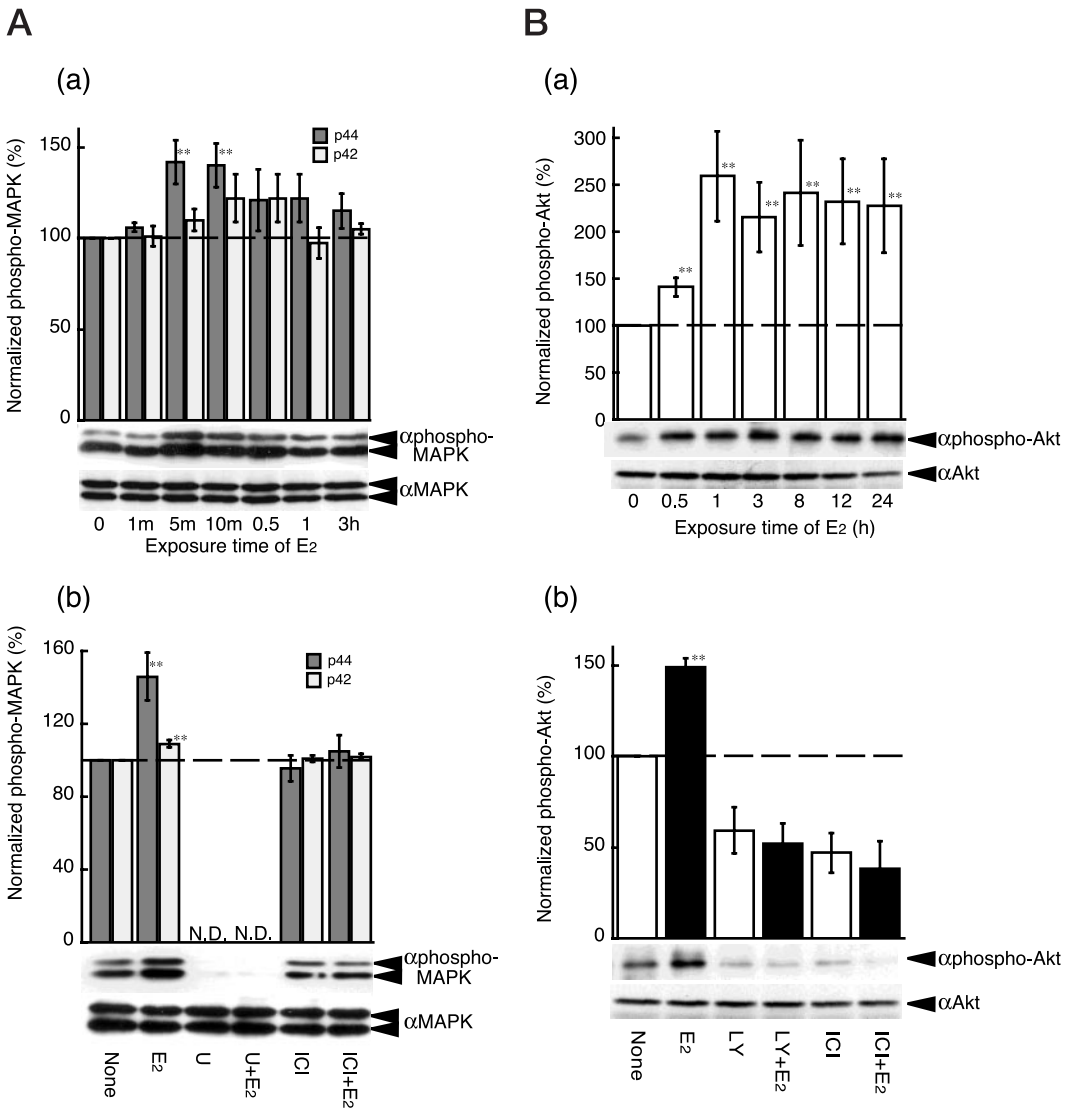

Fig. 3. Estradiol Caused the Phosphorylation of p44/42 MAPK and Akt

A, Immunoblotting with anti-phospho-p44/42 MAPK or p44/42 MAPK antibody, and quantification by densitometry. a, Estradiol activated p44/42 MAPK within 5 min. b, Activated MAPK by estradiol application for 5 min was inhibited by U0126 (10 μM) or ICI182,780 (10 μM). These results indicate that MAPK activation induced by estradiol occurred through the ER. Data represent the mean ± SEM (n = 5). **, $P < 0.01$ *vs.* control (none; Student's *t* test). *Upper band*, p44 MAPK; *lower band*, p42; N. D., not detected; ICI, ICI182,780; U, U0126. B, Immunoblotting with anti-phospho-Akt antibody or Akt antibody, and quantification by densitometry. a, Estradiol activated Akt (which is downstream of PI 3-kinase) within 30 min. b, Activated Akt by estradiol was inhibited by LY294002 (10 μM) or ICI182,780 (10 μM). Estradiol was applied for 1 h. These results indicate that Akt activation by estradiol is dependent on the activation of ER. Data represent the mean ± SEM (n = 6). **, $P < 0.05$ *vs.* control (none; Student's *t test*). LY, LY294002.

the HK^+-induced release of glutamate through potentiation of the exocytotic machinery.

Progesterone Decreased HK^+-Induced Glutamate Release

We tested the effect of other steroid hormones, progesterone and testosterone, on the HK^+-evoked release of glutamate. As shown in Fig. 8, progesterone did not affect the HK^+-induced glutamate release at 4 nM, but it significantly decreased the HK^+-induced release at 40 and 400 nM. Testosterone (0.1, 1, and 10 μM) did not alter the amount of glutamate released (data obtained at 10 μM are shown in Fig. 8), indicating that the potentiation of the release of glutamate was a β-estradiol-specific action.

DISCUSSION

Previous studies have shown that estradiol potentiated postsynaptic function. Chronic treatment with estradiol increases the dendritic spine density (22) and

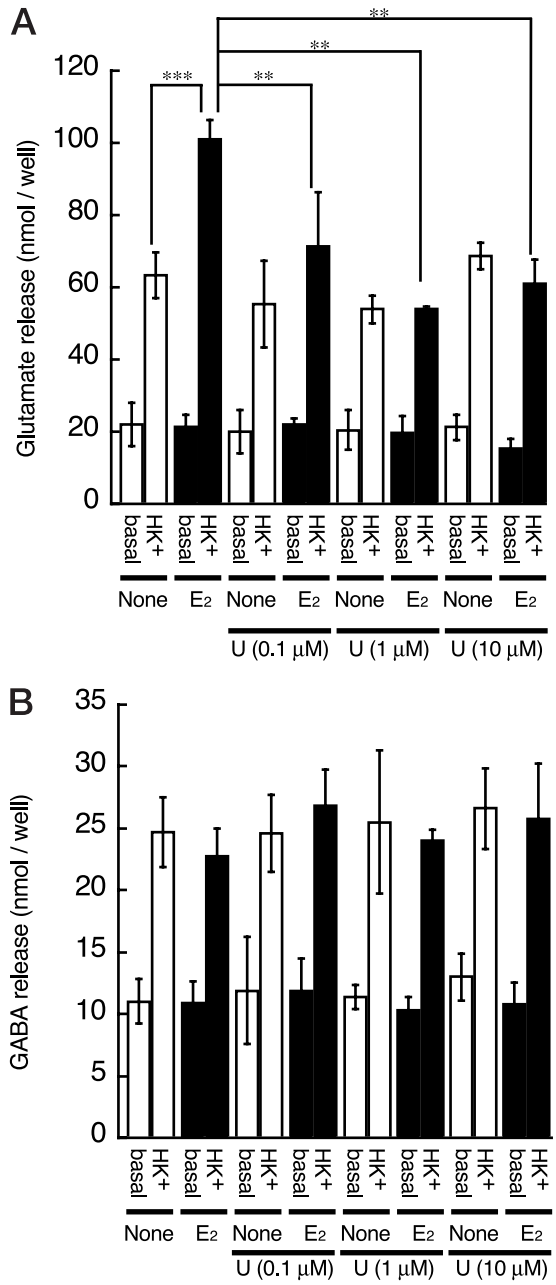

Fig. 4. MAPK Pathways Were Involved in Estradiol-Enhanced Glutamate Release

The effects of U0126 (MEK inhibitor; 0.1, 1, and 10 μM) were tested. A, U0126 completely blocked the effect of estradiol. This result suggests that estradiol exerts its effect via activation of MAPK. B, U0126 did not influence the HK^+-induced GABA release. Cells were cultured for 8 d *in vitro*. Data represent the mean ± SD (n = 3 or 4). **, $P < 0.01$ *vs.* E2-treated HK^+-evoked release; ***, $P < 0.01$ *vs.* unaffected HK^+-evoked release (Student's *t* test). E2, β-Estradiol (10 nM); U, U0126.

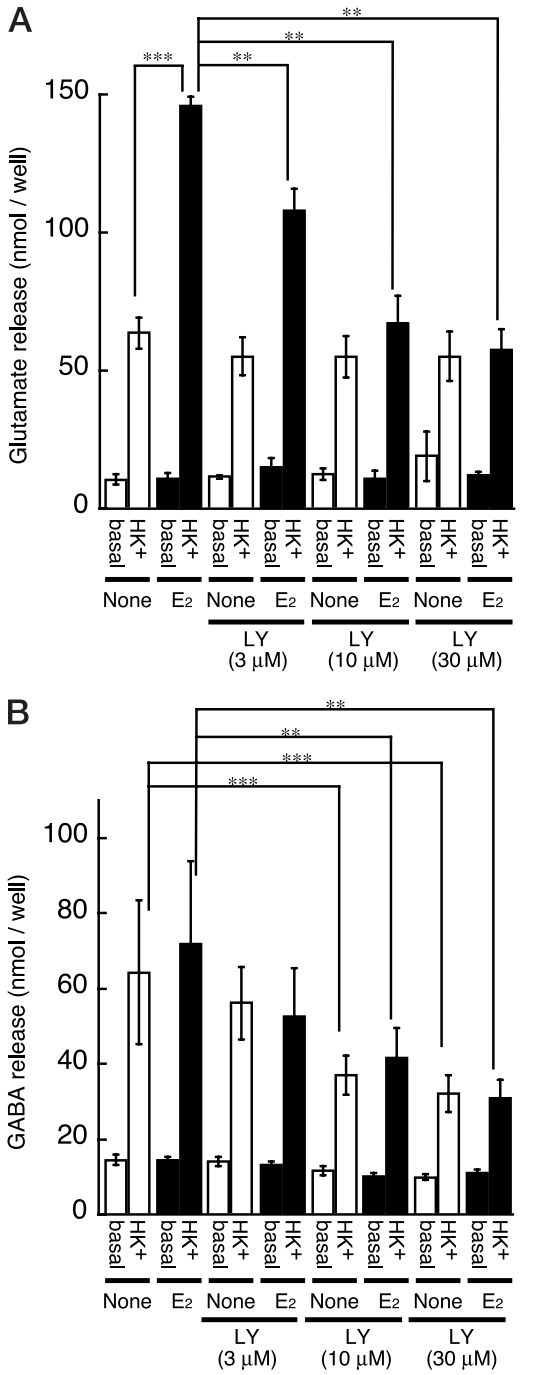

Fig. 5. PI 3-Kinase Pathways Were Involved in Estradiol-Enhanced Glutamate Release

The effects of LY294002 (a PI 3-kinase blocker) were tested. A, LY294002 inhibited the effect of estradiol in a dose-dependent manner, suggesting that estradiol exerted its effect via activation of PI 3-kinase. B, LY294002 blocked HK^+-induced GABA release in a dose-dependent manner, although enhanced release by estradiol was not observed. This result suggests that PI 3-kinase signaling is necessary for GABA release with or without estradiol treatment. Cells were cultured for 10 d *in vitro*. Data represent the mean ± SD (n = 3 or 4). **, $P < 0.01$ *vs.* E2-treated HK^+-evoked release; ***, $P < 0.01$ *vs.* unaffected HK^+-evoked release (Student's *t* test). E2, β-Estradiol (10 nM); LY, LY294002.

the number of NMDA receptor binding sites (23, 24) in the hippocampus CA1 region of ovariectomized rats. In this study, we found that estradiol reinforced synaptic efficacy via potentiation of the presynaptic func-

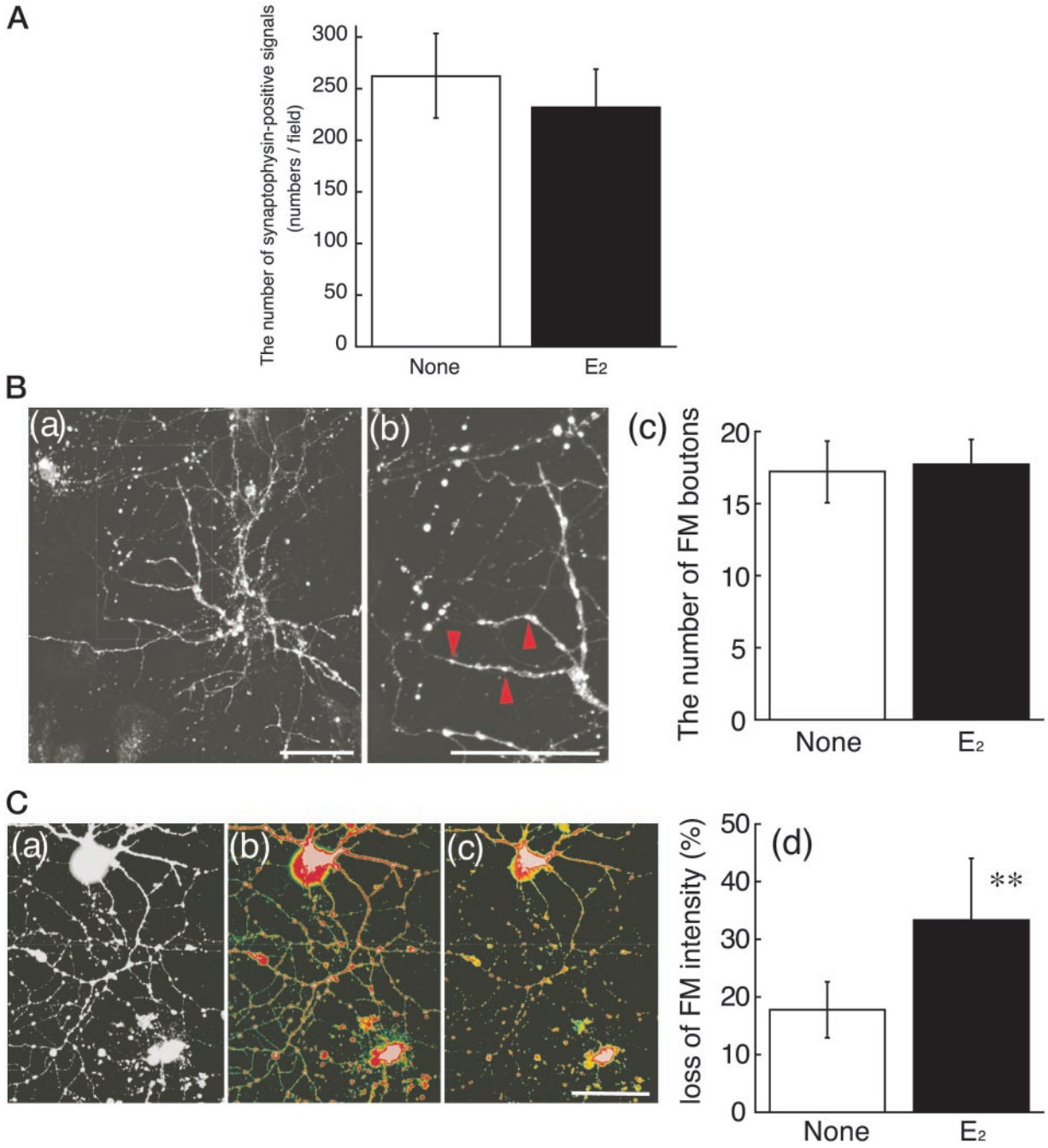

Fig. 6. Estradiol Did Not Change the Number of Presynaptic Sites but Activated the Exocytotic Machinery

A, The number of synaptophysin-immunopositive signals of immunocytochemical staining with antisynaptophysin antibody was not changed by estradiol. This result indicates that estradiol (10 nM for 24 h) did not alter the number of presynaptic sites. Data represent the mean ± SD (n = 10). E2, β-Estradiol. B: a, The representative image after FM1-43 staining in cultured hippocampal neurons (none-treated); b, the expanded image of squared *area of left panel*. Vesicle-like boutons containing FM1-43 dye were observed; c, the number of FM1-43 boutons in neurites in none-treated or estradiol-treated neurons. The number of boutons in neurites (per 50 μm) was counted. The number of FM boutons was not changed by estradiol treatment at 10 nM for 24 h. Data represent the mean ± SD (n = 10). N indicates the number of selected neurites. E2, β-Estradiol. C, Monitoring of FM1-43 fluorescent intensity before and after HK^+ (depolarization, 50 mM) stimulation. a, The monochrome image after FM1-43 staining in estradiol-treated neurons before HK^+ stimulation. b, The pseudocolor image before stimulation. c, The pseudocolor image after HK^+ stimulation. d, Increased loss of FM intensity after HK^+ stimulation was observed with estradiol pretreatment, suggesting that estradiol activated exocytotic machinery. The summarized data from six cells (two independent cultures) is shown. Data represent the mean ± SD (n = 20; n indicates the number of vesicle-like FM positive boutons). The FM loss was quantified by comparing the HK^+-stimulated intensity (60 sec after stimulation) with basal intensity (4 sec before stimulation). **, $P < 0.01$, *vs.* none-treated culture (Student's *t* test). E2, β-Estradiol (10 nM); *scale bar*, 50 μm.

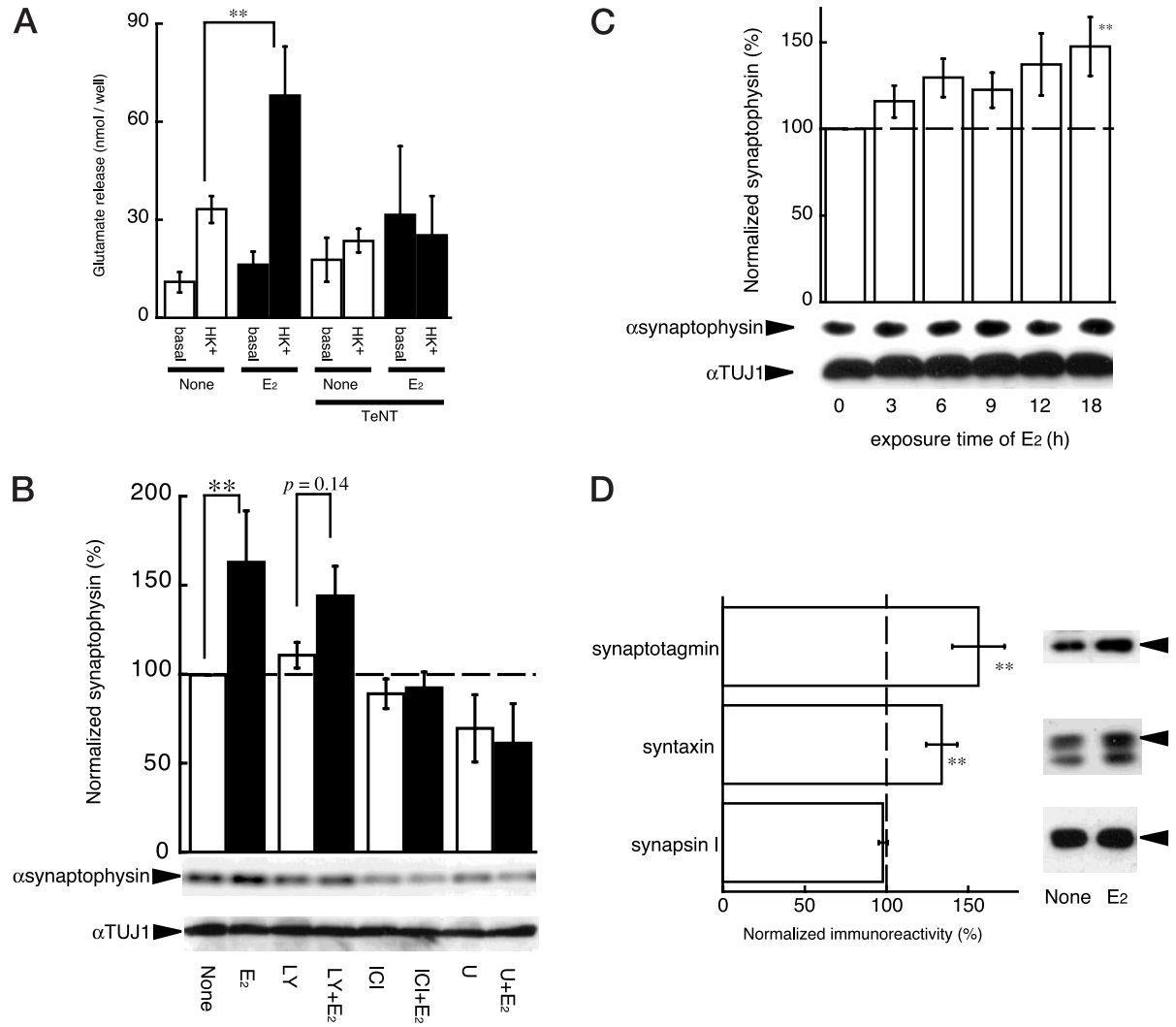

Fig. 7. Estradiol Facilitated the Glutamate Release through an Exocytotic Mechanism and Up-Regulated the Expression of Synaptophysin, Synaptotagmin, and Syntaxin

A, Estradiol-potentiated glutamate release was inhibited by pretreatment with TeNT. TeNT was applied to the cultured cells for 5 h at 10 nM. Cells were cultured for 7 d *in vitro*. Data represent the mean ± SD (n = 3 or 4). **, $P < 0.01$, *vs.* unaffected HK^+-evoked release (Student's *t* test). E2, β-Estradiol (10 nM, 24 h). B, Immunoblotting with antisynaptophysin antibody in cultured hippocampal neurons, and quantification by densitometry. Estradiol up-regulated the expression of synaptophysin, and this effect was completely blocked by ICI182,780 (10 μM) or U0126 (10 μM) and partially by LY294002 (10 μM; $P = 0.14$). It was confirmed by CBB staining and immunoblotting with anti-TUJ1 antibody that equal amounts of total protein were applied in each lane. Data represent the mean ± SEM (n = 6). **, $P < 0.05$ *vs.* control (none; Student's *t* test). E2, β-Estradiol (10 nM, 24 h); LY, LY294002; ICI, ICI182,780; U, U0126. C, Time course of synaptophysin expression by estradiol exposure. The up-regulation of expression of synaptophysin was observed within 18 h. Data represent the mean ± SEM (n = 5). **, $P < 0.05$ *vs.* control (0 h; Student's *t* test). E2, β-Estradiol (10 nM); TUJ1, class III β-tubulin. D, Estradiol up-regulated the expression of synaptotagmin and syntaxin. On the other hand, the expression level of synapsin I was not influenced by estradiol. The *lower band* of syntaxin may be a variant or degradation product of this protein. Data represent the mean ± SEM (n = 5). **, $P < 0.05$ *vs.* control (none; Student's *t* test). E2, β-Estradiol (10 nM, 24 h).

tion. We showed that chronic treatment with estradiol promoted depolarization-evoked glutamate release through the activation of PI 3-kinase and MAPK. It was also revealed that estradiol up-regulated the expression of synaptic proteins, which are required the exocytotic mechanism in the neurotransmitter release. These results suggest that estrogen has a crucial role in the presynaptic function in hippocampal neurons.

Estradiol enhanced the HK^+-induced glutamate release (Fig. 1A), and the estradiol-potentiated effect was inhibited by TeNT, which are known to block synaptic vesicle fusion (Fig. 7A), implying that the exocytotic system was up-regulated by estradiol. Furthermore, the imaging analysis with FM1-43 directly indicated that estradiol activated the exocytotic machinery (Fig. 6). Many reports suggested that pre-

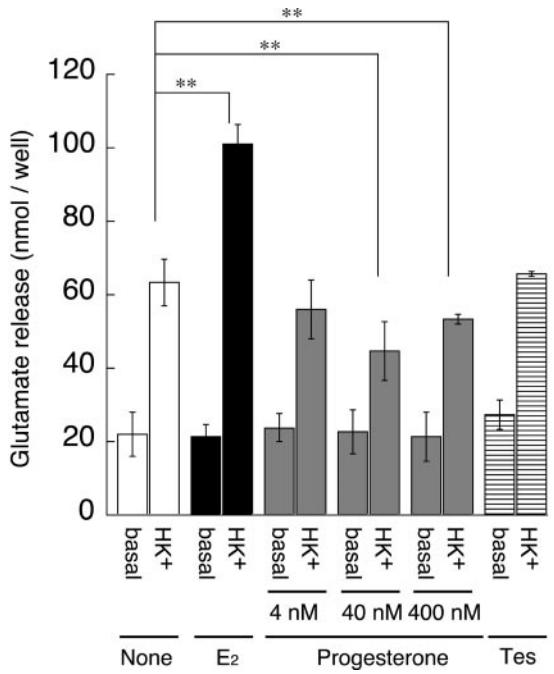

Fig. 8. The Effect of Progesterone and Testosterone on the HK^+-Induced Glutamate Release

Cultured hippocampal neurons were pretreated with progesterone at 4, 40, and 400 nM for 24 h. Progesterone did not affect the HK^+-evoked release at 4 nM, whereas it significantly decrease the release at 40 and 400 nM. Testosterone had no effect. Cells were cultured for 8 d *in vitro*. Data represent the mean ± SD (n = 3 or 4). **, $P < 0.01$, *vs.* unaffected HK^+-evoked release (Student's *t* test). E2, β-Estradiol (10 nM, 24 h); Tes, testosterone (10 μM, 24 h).

synaptic proteins were involved in the exocytotic machinery. For example, synaptophysin, synaptotagmin, and synapsin I are vesicle membrane proteins, and syntaxin localizes in presynaptic plasma membrane (38–39). Synaptotagmin interacts with plasma membrane protein (syntaxin, *etc.*) and calcium (acts as a calcium sensor), when synaptic vesicles were docking and during fusion (40). Synapsin I is a key molecule of vesicle cross-linking and anchoring to the actin filament in presynaptic terminals (41). Synaptophysin is considered important for the vesicle fusion system (exocytosis; Ref. 38). Presynaptic terminal protein, syntaxin, is also essential for the vesicle-plasma membrane docking process (42). Therefore, the control of these presynaptic protein expressions is expected to influence the amount of neurotransmitter release in the synaptic cleft and is thought to underlie some forms of synaptic plasticity (43–45). In this study, we found that the expression of presynaptic proteins (synaptophysin, synaptotagmin, and syntaxin) was up-regulated by chronic treatment of estradiol (Fig. 7, B and D). Murphy and Segal (7) demonstrated that estradiol increased the number of synaptophysin-containing boutons in cultured hippocampal neurons taken from 19-d-old rat embryos (application of estradiol at 0.1 μg/ml, approximately 400 nM, for 48 h). In our system (treatment with estradiol at 10 nM for 24 h), the FM1-43 staining and immunocytochemistry with antisynaptophysin antibody indicated that estradiol did not change the number of presynaptic sites (Fig. 6, A and B). However, estradiol up-regulated the expression of synaptophysin (Fig. 7B), suggesting that estradiol increased the expression of synaptophysin at individual synaptic sites. The longer treatment with estradiol in our culture may cause the increase in the number of presynaptic sites.

We examined the dose-dependent effect of estradiol on the glutamate release. Interestingly, 100 nM was not effective, whereas 1 and 10 nM were effective (Fig. 1D). The reason for this is not understood. However, it was reported that estradiol at high concentrations was not effective or had an opposite effect on therapy for anxiety (46) and on protection against lipopolysaccharide-induced cell death in microglia (47). The same phenomenon might occur in this study; that is, the dose of estradiol might be critical for the biological effects on the cells.

The effect of another ovarian hormone, progesterone, was examined. It was revealed that progesterone did not potentiate the HK^+-evoked glutamate release and significantly depressed the release (Fig. 8). It has been reported that progesterone has a physiological role in the potentiation of GABA-mediated synaptic transmission. Smith and colleagues (48, 49) showed that progesterone augmented GABA-responsiveness in Purkinje cells. They speculated that the Cl^- influx through the $GABA_A$ receptor was involved in the phenomenon. Therefore, it was supposed that the depression of the release of glutamate by progesterone in our study was a secondary effect through the modulation of the $GABA_A$ receptor. Another possibility is that progesterone potentiated the action of the endogenous inhibitory substance adenosine, which inhibits the neuronal transmission, because progesterone is known to decrease the uptake of adenosine (50–52).

The effect of estradiol on the release of glutamate was inhibited by the ER antagonists tamoxifen and ICI182,780 (described in *Results* and Fig. 2), suggesting that this effect required the activation of the ER. Activation of the nuclear ER was believed to be important for the neuronal effect of estradiol. In the hippocampus, expression of the nuclear ER (originally classified as ERα) is limited. In contrast, a novel nuclear ER (ERβ) is widely expressed in the hippocampus (53). In addition, it was recently suggested that estrogen acted through another pathway via a putative membrane-associated ER (54–56). This hypothesis was proposed from many results that estradiol rapidly activates MAPK or PI 3-kinase pathways (27–31). In the present study, we also observed that estradiol rapidly activated both PI 3-kinase and the MAPK pathway and confirmed that these activations were blocked by ICI182,780, an ER antagonist (Fig. 3). Therefore, the effects of estradiol on hippocampal neurons may require a membrane-associated ER, al-

though the involvement of the ERα or ERβ was not excluded in our study.

It has recently been reported that estradiol activates MAPK signaling. Watters *et al.* (27) reported that estradiol caused the phosphorylation of MAPK in the neuroblastoma cell line SK-N-SH. In addition, estradiol increased the activity of MAPK in adult rat hippocampus (28). In this study, estradiol significantly activated MAPK (Fig. 3A), and an inhibitor of the MAPK pathway [U0126, an inhibitor of MAPK kinase (MEK), which is a upstream molecule of MAPK] blocked the estradiol-potentiated glutamate release (Fig. 4A), suggesting that the estradiol-potentiated release was dependent on the activation of MAPK. Furthermore, we found that the increase in expression of synaptophysin required the activation of MAPK. The involvement of MAPK activation in synaptic plasticity and memory function has recently been reported (57, 58). Therefore, MAPK activation by estradiol is considered important for neuronal plasticity.

PI 3-kinase has widely been implicated as an intracellular transducer of survival signals initiated by various growth factors (59–62). Several studies indicate that estradiol exerts biological effects via activation of PI 3-kinase. Simoncini *et al.* (29) reported that estradiol increased the activity of endothelial nitric oxide synthase through the activation of PI 3-kinase. Their study showed that ERα binds to the p85α regulatory subunit of PI 3-kinase in a ligand-dependent manner. It was also shown that estradiol activated PI 3-kinase in cultured cortical neurons and protected neurons from glutamate-induced neurotoxicity (30). We confirmed that estradiol activated PI 3-kinase signaling (Fig. 3B), and the estradiol-potentiated release of glutamate was inhibited by LY294002 (PI 3-kinase inhibitor) in a dose-dependent manner (Fig. 5A), indicating that the activation of PI 3-kinase was necessary for this effect. Interestingly, the HK^+-evoked GABA release with or without estradiol treatment was reduced by LY294002, although potentiation of HK^+-evoked GABA release by estradiol was not observed (Fig. 5B). These results indicate the possibility that a PI 3-kinase signaling pathway participates in the acute modulation of presynaptic function. The increase in expression of synaptophysin was also inhibited by LY294002, but this inhibitory effect was partial (Fig. 7B), implying that PI 3-kinase was involved not only in the regulation of expression of synaptic protein but also in other synaptic systems. For example, PI 3-kinase possibly participates in the synthesis of neurotransmitter (for instance, the expression of the glutamate synthesis enzyme, glutaminase, was regulated by estradiol; our unpublished data), although further investigation is needed. Recently, it was suggested that PI 3-kinase was involved in the generation of long-term potentiation in the hippocampal dentate gyrus (63) and amygdala (64). For example, Lin *et al.* (64) indicated that PI 3-kinase was an important molecule for consolidation of fear memory. These studies suggest that PI 3-kinase is important for neuronal plasticity, although it is still unclear how PI 3-kinase regulates the system of neurotransmitter release. Yang *et al.* (65) have shown that both PI 3-kinase and phospholipase C-γ pathways (which induces the release of Ca^{2+} from intracellular stores) were necessary and sufficient to mediate neurotrophin-3-induced synaptic potentiation (MAPK was not necessary). Furthermore, Amino *et al.* (66) showed the necessity of both PI 3-kinase and the MAPK pathways in nerve growth factor-enhanced dopamine release. All these findings (including our study) suggest the possibility that activations of several pathways are required for the potentiation of transmitter release.

In the present study, we found that estradiol potentiated the release of glutamate induced by HK^+ stimulation through the up-regulation of exocytotic machinery and that the concentration of estradiol is critical for this phenomenon. Does estrogen actually regulate synaptic transmission *in vivo*? As an example, the neuronal physiology of the female rat is known to fluctuate across the estrous cycle (67–69). In male rats, estradiol is synthesized from testosterone by aromatase P450 (70), which is expressed in various regions of the brain (71), implying that estradiol was also effective in male brain. Sex steroids influence spatial memory, fear-related memory, and emotional behavior. For instance, it has been demonstrated that estrogen exerts energizing and antidepressant effects (72–75), and progesterone, in contrast, inhibits kindling and seizure activity (46, 76–78). Furthermore, estrogen facilitates working memory in the radial-arm maze test and water maze task (79, 80). Thus, although further examination is required, all these findings, including our own, indicate that ovarian steroids are an important factor in learning, memory, and emotional behavior.

MATERIALS AND METHODS

Cell Preparation

Cell cultures were prepared from 2- to 3-d-old rats (Wistar ST; SLC Co., Shizuoka, Japan) as reported previously (81–84). Brains were removed and collected in ice-cold (4 C) L15 medium (Life Technologies, Inc., Rockville, MD) supplemented with 0.6% glucose. Then, the hippocampus were dissected and mechanically dissociated with a plastic pipette after digestion with papain (90 U/ml; Worthington Biochemical Corp. Co., NJ) at 37 C for 20 min. The dissociated cells were plated at a final density of 4–5 $\times$ $10^5/cm^2$ on polyethyleneimine-coated 12- and 24-well plates (3.8- and 2-cm^2 surface area/well, respectively; Falcon; Becton Dickinson, Labware, Franklin Lakes, NJ). The culture medium (DF medium) consisted of 5% (vol/vol) precolostrum newborn calf serum (Mitsubishi Kasei Co., Tokyo, Japan), 5% (vol/vol) heat-inactivated horse serum (Life Technologies, Inc.), and 90% of a 1:1 mixture of DMEM (Life Technologies, Inc.) and Ham's F-12 medium (Life Technologies, Inc.) containing 15 mM HEPES buffer (pH 7.4), 30 nM Na_2SeO_3, and 1.9 mg/ml of $NaHCO_3$. After 24 h, the culture medium was replaced with charcoal-treated precolostrum newborn calf serum and horse serum (both at 5%; charcoal treatment was performed

to remove estrogen-like substances in serum) and DF medium (90%). The cultures were maintained for 7–10 d.

Chemicals

β-Estradiol, α-estradiol, progesterone, and testosterone were purchased from Sigma (St. Louis, MO). Tamoxifen and ICI182,780 were obtained from Tocris Cookson Inc. (St. Louis, MO). U0126 was obtained from Promega Corp. (Madison, WI). LY294002 was purchased from Calbiochem-Novabiochem GmbH (Schwalbach, Germany). Steroid hormones were applied for 24 h before the assay. Inhibitors (tamoxifen, ICI182,780, U0126, and LY294002) were added 2 h before the application of estradiol and treated during estradiol administration (26 h in total).

Immunocytochemistry

Cells were stained with antimicrotubule-associated protein 2 (MAP2) and antisynaptophysin antibodies. Briefly, cells were fixed in 4% paraformaldehyde at room temperature for 20 min, before incubation with the anti-MAP2 antibody (a gift from Dr. H. Murofushi, The University of Tokyo) diluted 1:1000 with a solution containing 3% nonfat dried milk and 0.03% Triton X-100 at 4 C for 12 h. To visualize, we used a Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA) with 0.02% (wt/vol) 3,3′-diaminobenzidine tetrahydrochloride, and 0.1% (wt/vol) $(NH_4)Ni(SO_4)_2$ dissolved in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.01% (vol/vol) H_2O_2. For staining with the antisynaptophysin antibody (Boehringer Mannheim GmbH, Mannheim, Germany), cells were treated with 0.25% Triton X-100 for 5 min. Then, the first antibody was used at a dilution of 1:100. Fluorescein isothiocyanate-labeled antimouse antibody (1:100) was used as a secondary antibody, and the cells were observed by fluorescent microscopy (TMD-300, Nikon, Tokyo, Japan).

Detection of Amino Acid Neurotransmitters

The amounts of amino acids from the cultured hippocampal neurons were measured as described previously (82–84). Briefly, the amounts of amino acids released into the assay buffer [modified HEPES-buffered Krebs Ringer solution (KRH) containing 130 mM NaCl, 5 mM KCl, 1.2 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 10 mM glucose, 1% BSA, and 25 mM HEPES (pH 7.4)] from the cultured neurons were measured by HPLC (Shimadzu Co., Kyoto, Japan) and fluorescence detector (RF10A-xl; Shimadzu Co.). Before the samples were collected, the culture medium was replaced with serum-free medium for 4–5 h. Then, cultured neurons were washed three times with KRH buffer. The HK^+ solution consisted of 85 mM NaCl, 50 mM KCl, 1.2 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 10 mM glucose, 1% BSA, and 25 mM HEPES (pH 7.4). Fractions were collected into tubes on ice by the batch method every 3 min and filtered with 0.22-μm membranes to remove cell debris. These operations were carried out under the control of temperature at 37 C. Amino acids in the assay buffer were treated with *O*-phthalaldehyde and 2-mercaptoethanol for 5 min at 15 C. Samples were then injected into the HPLC system and analyzed using a fluorescence monitor (excitation wavelength, 350 nm; emission wavelength, 450 nm). Nifedipine (10 μM; Sigma) was applied to the cells for 30 min before the assay, and the samples were collected in the presence of this drug. TeNT (10 nM; List Biological Laboratories, Inc., Campbell, CA) was applied to the cells for 5 h before the samples were collected. All experiments were performed in 4–7 separated cultures to confirm whether the obtained data were reproducible. Representative data from a sister culture are shown in the figures. N in figure legends indicates the number of wells in one plate.

FM1-43 Imaging

The imaging analysis was performed as described previously (82, 84). Briefly, the cultured hippocampal neurons were maintained on polyethyleneimine-coated glasses (Matsunami, Osaka, Japan) attached to flexiperm (IN VITRO, Kalkberg, Germany). After several washes with KRH (containing 5 mM KCl), the indicator dye FM 1–43 (2 μM) was loaded into living cells by incubating with HK^+ loading solution (KRH containing 50 mM KCl) for 10 min at 37 C. After several washes with KRH (5 mM KCl), the dye intensity was observed using a confocal laser microscope (RCM 8000; Nikon, Tokyo, Japan). Cells were irradiated with an excitation blue light beam (488 nm) produced by an argon ion laser. The emitted fluorescence was guided through a $\times$20 water-immersion objective to a pinhole diaphragm at 520 nm. Then, the number of the FM1-43-positive signals in selected neurites (per 50 μm) were counted. Next, the time-course analysis of reduction in FM fluorescence intensity induced by HK^+ solution (at the final concentration of 50 mM) was performed. The intensity of FM emission was scanned at a dwell time of 1/30 sec with a monitor video enhancer, and image data were obtained every 4 sec. The image data were stored in an RCM workstation and analyzed. The loss of fluorescence intensity was determined by comparing the stimulation intensity (60 sec after HK^+ stimulation) with basal intensity (4 sec before stimulation). FM dye fluorescence intensities were confirmed as stable with minimum bleaching for more than 3 min in vehicle solution. To confirm reproducibility, the imaging experiment was performed four times in independent cultures. Representative data from six cells are shown in Fig. 6.

Immunoblotting

Cells were lysed in sodium dodecyl sulfate (SDS) lysis buffer containing 1% SDS, 20 mM Tris-HCl (pH 7.4), 5 mM EDTA (pH 8.0), 10 mM NaF, 2 mM Na_3VO_4, 0.5 mM phenylarsine oxide, and 1 mM phenylmethylsulfonyl fluoride. The lysates were boiled for 3 min and then clarified by ultracentrifugation at 60,000 $\times$ *g* for 30 min at 8 C. The protein concentration of the supernatants was determined using a BCA protein assay kit (Pierce Chemical Co., Rockford, IL), and then 10-μg aliquots of protein were resolved on electrophoresison 10% SDS-polyacrylamide gels. Proteins were transferred onto polyvinylidene fluoride membranes (Millipore Corp., Bedford, MA) in 0.1 M Tris base, 0.192 M glycine, and 20% methanol using a semi-dry electrophoretic transfer system. The membranes were blocked overnight at 4 C with 0.1% Tween 20/Tris-buffered saline (T-TBS) containing 5% nonfat dried milk. Membranes were then probed with several primary antibodies as follows: 1:250 dilution of antisynaptophysin antibody (Boehringer Mannheim GmbH); 1:1000 anti-class III β-tubulin (TUJ1; Berkeley Antibody Co., Richmond, CA); 1:500 anti-synaptotagmin (Transduction Laboratories, Lexington, KY); 1:10000 anti-syntaxin (Sigma); 1:2000 anti-synapsin I (Chemicon International, Temecula, CA); 1:1000 anti-phospho-p44/42 MAPK antibody (Cell Signaling Technology Inc., Beverly, MA); 1:1000 anti-p44/42 MAPK antibody (Cell Signaling Technology Inc.); 1:1000 anti-phospho-Akt antibody (Cell Signaling Technology Inc.); or 1:1000 anti-Akt antibody (Cell Signaling Technology Inc.). These antibodies were reacted in T-TBS containing 1% nonfat dried milk or 5% BSA at room temperature for 1 h. After several washes with T-TBS, the membranes were incubated with horseradish peroxidase-conjugated donkey antimouse IgG or goat antirabbit IgG secondary antibody (Zymed Laboratories, Inc., San Francisco, CA) diluted 1:2000 with T-TBS at room temperature for 1 h. They were then washed at least 4 times with T-TBS and visualized using the ECL chemiluminescence system (Immunostar; Wako, Osaka, Japan). In addition to immunoblotting, Coomassie brilliant blue (CBB) staining was performed to detect the total amount of protein. For CBB staining, poly-

acrylamide gels, after the protein was transferred to membranes, were shaken with a solution containing 0.1% CBB, 10% acetic acid, 30% methanol, and 59.9% H_2O, then stained gels were washed with a solution containing 10% acetic acid, 30% methanol, and 60% H_2O. To quantify the amount of proteins after Western blot analysis, we measured the density of immunoblots with image-analysis software (Science Lab 98 Image Gauge; Fuji Photo Film Co., Ltd., Tokyo, Japan). The changes in protein levels were expressed as a percentage of controls, respectively.

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