Molecular Pain

Research

Glycine receptor in rat hippocampal and spinal cord neurons as a molecular target for rapid actions of 17-β-estradiol

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Abstract

Glycine receptors (GlyRs) play important roles in regulating hippocampal neural network activity and spinal nociception. Here we show that, in cultured rat hippocampal (HIP) and spinal dorsal horn (SDH) neurons, 17-β-estradiol (E_2) rapidly and reversibly reduced the peak amplitude of whole-cell glycine-activated currents (I_{Gly}). In outside-out membrane patches from HIP neurons devoid of nuclei, E_2 similarly inhibited I_{Gly}, suggesting a non-genomic characteristic. Moreover, the E_2 effect on I_{Gly} persisted in the presence of the calcium chelator BAPTA, the protein kinase inhibitor staurosporine, the classical ER (i.e. ERα and ERβ) antagonist tamoxifen, or the G-protein modulators, favoring a direct action of E_2 on GlyRs. In HEK293 cells expressing various combinations of GlyR subunits, E_2 only affected the I_{Gly} in cells expressing α2, α2β or α3β subunits, suggesting that either α2-containing or α3β-GlyRs mediate the E_2 effect observed in neurons. Furthermore, E_2 inhibited the GlyR-mediated tonic current in pyramidal neurons of HIP CA1 region, where abundant GlyR α2 subunit is expressed. We suggest that the neuronal GlyR is a novel molecular target of E_2 which directly inhibits the function of GlyRs in the HIP and SDH regions. This finding may shed new light on premenstrual dysphoric disorder and the gender differences in pain sensation at the CNS level.

Background

Studies over the last several decades have demonstrated that estrogen plays an important role in not only reproduction, but also regulation of early CNS development [1] and in synaptic plasticity of the mature hippocampus [2]. The classical estrogen actions in the CNS are primarily mediated by activating nuclear estrogen receptor α and β (ERα/β), causing long-term genomic effects [3,4]. Nevertheless, it is becoming increasingly clear that estrogen can

activate cytoplasmic signaling events at or near the plasma membrane [5,6], presumably through either membrane-localized classical ERs [7,8] or novel ERs [9-11]. Moreover, estradiol is reported to directly bind to and modulate certain ion channels, like the Maxi-K channels [12], indicating the existence of additional estrogen targets besides ERs. In the hippocampus, both *in vivo* [13] and *in vitro* [14-16] studies have focused on the inhibitory GABAergic machineries, and suggested that estradiol alters neuronal

activity by suppressing GABAergic synaptic transmission. A recent study also indicated that estradiol inhibits human recombinant rho1 $GABA_C$ receptor [17].

Like $GABA_A$ receptors ($GABA_A$Rs), the major receptor mediating central inhibition, GlyRs contribute to neuronal inhibition in hippocampus [18-20] and spinal cord [21,22]. GlyRs are pentamers and composed of $\alpha(1-4)$ and β subunits [21]. In hippocampal (HIP) neurons, GlyRs are thought to be primarily the homopentamer of $\alpha2$ subunits that function extrasynaptically to produce tonic inhibition [21]. Tonic activation of GlyRs leads to cross-inhibition of $GABA_A$Rs [23], and influences synaptic activity [18,24,25] and short-term plasticity [19]. In adult spinal dorsal horn (SDH), GlyRs are important in regulating nociception and motor function. For example, $\alpha3$-containing GlyRs regulate inflammatory pain sensitization [26]. Interestingly, during the development of the spinal cord, there is a switch of GlyR subunit composition from $\alpha2$ in the fetus to $\alpha1$ predominance in the adult [21,27], suggesting a role of the $\alpha2$ subunit in neuronal development. Indeed, two recent studies showed that GlyRs play an important role in rod photoreceptor development of the vertebrate retina [28] and regulate spinal interneuron differentiation in zebrafish [29]. On the other hand, estrogen is locally synthesized in the CNS [30] and the level of estrogen is under regulation [1,31]. A recent study showed that estradiol enhances the spontaneous synaptic release of glycine in hypoglossal motoneurones [32]. However, the estradiol effects on GlyRs remain unexplored. In this study, therefore, we examined the modulatory effects of 17-β-estradiol (E_2), the most prevalent and potent form of endogenous estrogen, on native GlyRs in HIP and SDH neurons, and on recombinant GlyRs expressed in HEK293 cells. This study will add a new dimension for understanding the multifaceted estrogenic effects in the CNS.

Results
17- -estradiol rapidly inhibits glycine-activated current (I_{Gly}) in cultured rat SDH and HIP neurons
At a holding potential (V_H) of -50 mV under whole-cell voltage clamp, application of glycine (100 μM) to the cultured HIP or SDH neurons elicited an inward current. The strychnine sensitivity and chloride dependence of the I_{Gly} suggests that it was mediated by GlyR-chloride channels (data not shown). After recording a stable control I_{Gly}, we pre-superfused the neurons with E_2 at various concentrations for 30 s, and then recorded I_{Gly} in the presence of E_2. The peak amplitude of I_{Gly} was rapidly reduced by E_2 application (Figure 1A), while it was not further inhibited when the pre-perfusion time was prolonged (data not shown). In neurons derived from neonatal rats of both sexes, E_2 exerted a similar inhibitory effect on I_{Gly}, therefore data from both sexes were pooled for comparison. As

shown in Figure 1B, E_2 concentration-dependently inhibited the peak I_{Gly}. On average, E_2 at 1, 3, 6 and 10 μM significantly reduced peak I_{Gly} to 95.0 ± 0.8% ($P < 0.05$ compared with control, n = 4), 85.8 ± 2.2% ($P < 0.01$ compared with control, n = 5), 80.2 ± 4.0% ($P < 0.01$ compared with control, n = 5) and 63.4 ± 2.2% ($P < 0.001$ compared with control, n = 8, Paired Student's *t*-test for all) of control in HIP neurons, respectively; and in SDH neurons, peak I_{Gly} was reduced to 97.0 ± 0.9% ($P < 0.05$ compared with control, n = 6), 89.1 ± 2.0% ($P < 0.01$ compared with control, n = 7), 83.1 ± 3.0% ($P < 0.01$ compared with control, n = 8) and 75.3 ± 2.1% ($P < 0.01$ compared with control, n = 7, $P < 0.01$ compared with the inhibition of 10 μM E_2 produced in HIP neuron) of control, respectively. The IC_{50} values of E_2 for I_{Gly} of the HIP neuron and SDH neuron are 16.5 ± 2.7 μM and 33.2 ± 3.4 μM ($P < 0.01$ compared with that in HIP neuron), respectively. Therefore E_2 has stronger inhibitory effect on I_{Gly} mediated by hippocampal GlyR. To assess possible contaminations of endogenous steroids derived from glial cells [30,33], we examined the E_2 effect in cultures grown without blocking the proliferation of glial cells and found that E_2 still inhibited I_{Gly} under such a condition (data not shown).

The concentration-effect relationships for E_2 shown in Figure 1B indicate that E_2 inhibited I_{Gly} more markedly in cultured HIP neurons. In the following experiments, therefore, we used HIP neurons to study the mechanisms underlying E_2 inhibition. To explore whether a genomic mechanism is responsible for E_2 inhibition on I_{Gly}, we first examined the effect of E_2 in large outside-out patches excised from cultured HIP neurons. The patches were exposed to rapid changes of glycine or glycine plus E_2. As shown in Figure 1C, the peak amplitude of I_{Gly} was significantly inhibited by E_2 (59.2 ± 3.6% of control, $P < 0.001$, Paired Student's *t*-test). Since outside-out patches contain no cellular nuclei, this result indicates that E_2 inhibited I_{Gly} via a non-genomic mechanism. We next examined the concentration-response relationships of I_{Gly} in the absence or presence of E_2. As shown in Figure 1D, E_2 at 10 μM suppressed I_{Gly} evoked by both subsaturating and saturating concentrations of glycine. The EC_{50} and Hill coefficient of glycine were 42.9 ± 4.3 μM and 1.7 in the absence of E_2 and 61.7 ± 8.1 μM and 1.2 in the presence of E_2, respectively. This result suggests that E_2 inhibits I_{Gly} in a noncompetitive manner.

No involvement of intracellular signaling pathways and classical ERs
Previous studies have indicated that the acute effect of E_2 occurring within a time course of milliseconds to minutes are attributed to the activation of intracellular signaling pathways mediated by presumably membrane-bound classical ERs [7,8,34,35] or novel ERs [9,10]. Additionally,
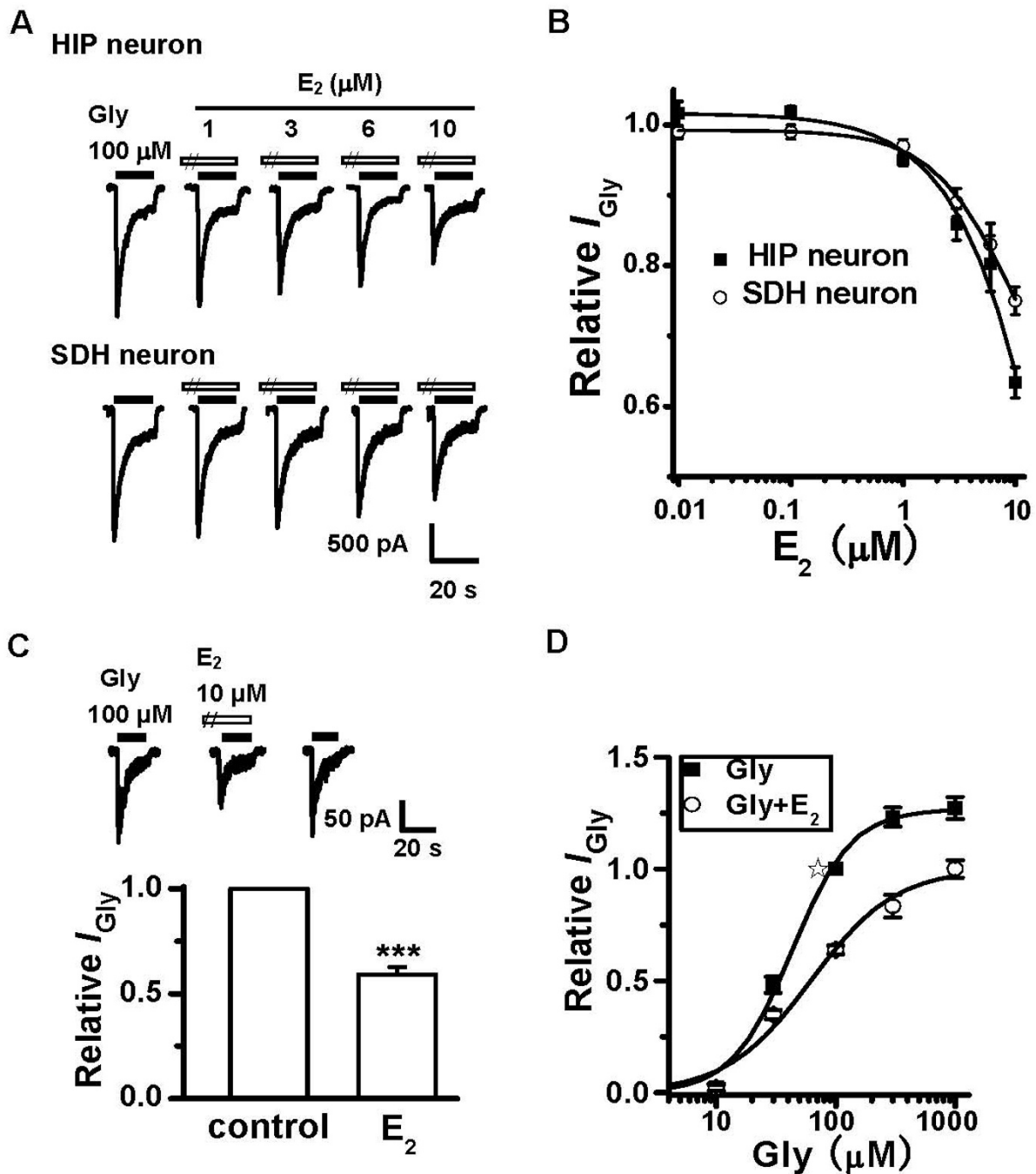
Figure 1
E_2-induced inhibition of I_{Gly} in cultured SDH and HIP neurons. (A) Representative traces of current induced by 100 μM glycine in the presence or absence of E_2 at various concentrations in cultured HIP (upper) and SDH (bottom) neurons. The neurons were pre-treated with E_2 for 30 s before E_2 and glycine were co-applied. (B) Summarized data illustrating the concentration dependence of E_2 inhibition (n = 4–8) as shown in *A*. (C) E_2 significantly inhibited I_{Gly} recorded from the outside-out patches (n = 5). The upper traces show the representative I_{Gly} recorded from outside-out patches in the presence and absence of E_2. ***P < 0.001, Paired Student's *t*-test, compared with control without adding E_2. (D) The concentration-response curves of I_{Gly} in the presence and absence of 10 μM E_2. For each neuron recorded, the current was normalized to the peak amplitude of I_{Gly} induced by 100 μM glycine alone (P) from the same neuron and each point represents the average value of 5–9 neurons.

E_2 can modulate calcium channels and affect the intracellular calcium level [36,37]. To explore the possible involvement of any intracellular pathways in mediating E_2 inhibition of I_{Gly}, following experiments were conducted. We first examined the role of the intracellular Ca^{2+}. When neurons were loaded with 15 mM BAPTA via the recording pipette, E_2 reduced the peak I_{Gly} to 62.7 ± 2.0% of the control, which was not significantly different from that obtained in the absence of BAPTA (Figure 2A1 and 2B1, P > 0.05, Unpaired Student's t-test). In order to test the role of protein phosphorylation and dephosphorylation in E_2 inhibition, we loaded the neurons with staurosporine (5 μM), a nonselective protein kinase inhibitor, to disrupt the balance between phosphorylation and dephosphorylation. Likewise, the inhibitory effect of E_2 on I_{Gly} was not altered (Figure 2A2 and 2C1, P > 0.05, Unpaired Student's t-test). A previous study [38] demonstrated that the GlyR is a target of the G protein βγ dimer. To examine the role of G proteins, we loaded the neurons with GTP-γ-S (500 μM) or GDP-β-S (500 μM) to activate or block the G protein, respectively. Neither of these treatments affected the inhibition induced by E_2 on I_{Gly} (Figure 2A3, A4, and 2C, P > 0.05, Unpaired Student's t-test). Thus, it is unlikely that E_2 exerts its inhibition on I_{Gly} through intracellular signaling pathways.

We further employed tamoxifen, a classical ER antagonist in the hippocampus [15], to examine whether membrane-localized classical ERs were involved in E_2 inhibition of I_{Gly}. After incubation of 1 μM tamoxifen for 2 h, we examined the effects of E_2 on I_{Gly} in the continued presence of tamoxifen. Though, consistent with the previous study [32], the peak amplitude of I_{Gly} induced by 100 μM glycine was decreased after the treatment with tamoxifen (Figure 2A5, note the difference in the scale bar), the inhibitory effect of E_2 on I_{Gly} was not affected (Figure 4A5 and 4B1, P > 0.05, Unpaired Student's t-test). Moreover, 17-α-estradiol (17-α-E_2, 10 μM), the inactive stereoisomer of E_2, mimicked the inhibitory effect of E_2 on I_{Gly} (Figure 2A6 and 2B1, P > 0.05, Unpaired Student's t-test), suggesting that E_2 inhibition on GlyR is independent of classical ERs.

Regulatory sites for E_2 and pregnanolone on GlyRs are separate
A previous study showed that another neurosteroid, pregnanolone (PGN) directly inhibited I_{Gly} in a competitive manner [39]. We were interested to know whether E_2 and PGN share a common binding site on GlyRs. If the sites are separate, the inhibitory effects should be additive when E_2 and PGN were co-applied. As shown in Figure 3A, PGN at 1 μM and 10 μM significantly inhibited the peak amplitude of I_{Gly} by 25.1 ± 7.6% and 49.0 ± 5.9% of control, respectively. In the presence of 10 μM E_2, additional inhibition of I_{Gly} was observed by PGN at both 1 μM (Figure 3B, 51.3 ± 5.3% of control), and 10 μM (Figure 3B, 70.4 ± 7.3% of control). These data suggest that distinct binding sites may mediate the inhibition of E_2 and PGN on I_{Gly}.

Subunit selectivity of E_2 inhibition on GlyRs
To determine which GlyR subunits are responsible for the E_2-induced inhibition, we investigated the E_2 effects on I_{Gly} in HEK293 cells expressing various recombinant GlyRs (Figure 4A). In GlyR subunit-untransfected cells, no glycine responses were observed (data not shown). As shown in Figure 4, E_2 selectively inhibited the peak I_{Gly} mediated by homomeric α2-GlyRs to 75.1 ± 4.3% of control (P < 0.001, n = 6, Paired Student's t-test), but had no significant effect on homomeric α1- and α3-GlyRs (99.8 ± 3.1% of control and 99.0 ± 1.2% of control for α1- and α3-GlyRs, respectively, P > 0.05, n = 10, Paired Student's t-test). To further examine whether co-expression of β subunit affected the E_2 inhibition, we tested the inhibitory effect of E_2 on heteromric GlyRs. We found that E_2 inhibited the peak I_{Gly} mediated by α2β – and α3β-GlyRs to 78.9 ± 2.9% (P < 0.001, n = 9, Paired Student's t-test) and 71.0 ± 3.0% of control (P < 0.001, n = 11, Paired Student's t-test), respectively. However, I_{Gly} mediated by α1β-GlyRs was not significantly affected. Thus, it is likely that either α2- or α2β – or α3β-GlyRs mediate the E_2 effect in SDH and HIP neurons.

The developmental changes of E_2 inhibition of GlyRs in spinal cord neurons
The GlyR subunit expression is developmentally regulated in some areas of CNS such as spinal cord. To test whether E_2 effects are different in different times in culture, we investigate the effects of E_2 on GlyR in spinal cord and HIP neurons from different days in culture. We first recorded I_{Gly} in cultured HIP neurons after different days of *in vitro* (DIV) differentiation. As shown in Figure 5, the inhibitory extent of E_2 on I_{Gly} did not change with time *in vitro* (P > 0.05, comparing DIV6–8, 13–15 and 20–23, n = 5–7, one-way ANOVA). We next examined the effects of E_2 on I_{Gly} induced by 100 μM glycine of spinal cord neurons in cultures after DIV6–8, 13–15 and 20–23. Interestingly, E_2 significantly inhibited I_{Gly} of the neurons at DIV6–8 and 13–15, but the inhibitory effects declined in the neurons at DIV20–23 (P = 0.012, comparing between DIV20–23 and DIV6–8; P = 0.023, comparing between DIV20–23 and DIV13–15. n = 6–8, one-way ANOVA).

Inhibitory effect of E_2 on GlyR-mediated tonic current in HIP slices
In the developing and mature hippocampus, α2 subunit represents the primary component of the functional GlyRs [21]. This unique property enables us to examine the action of E_2 on GlyRs from pyramidal neurons in CA1 region of HIP slices. Glycine concentration in cerebrospi-
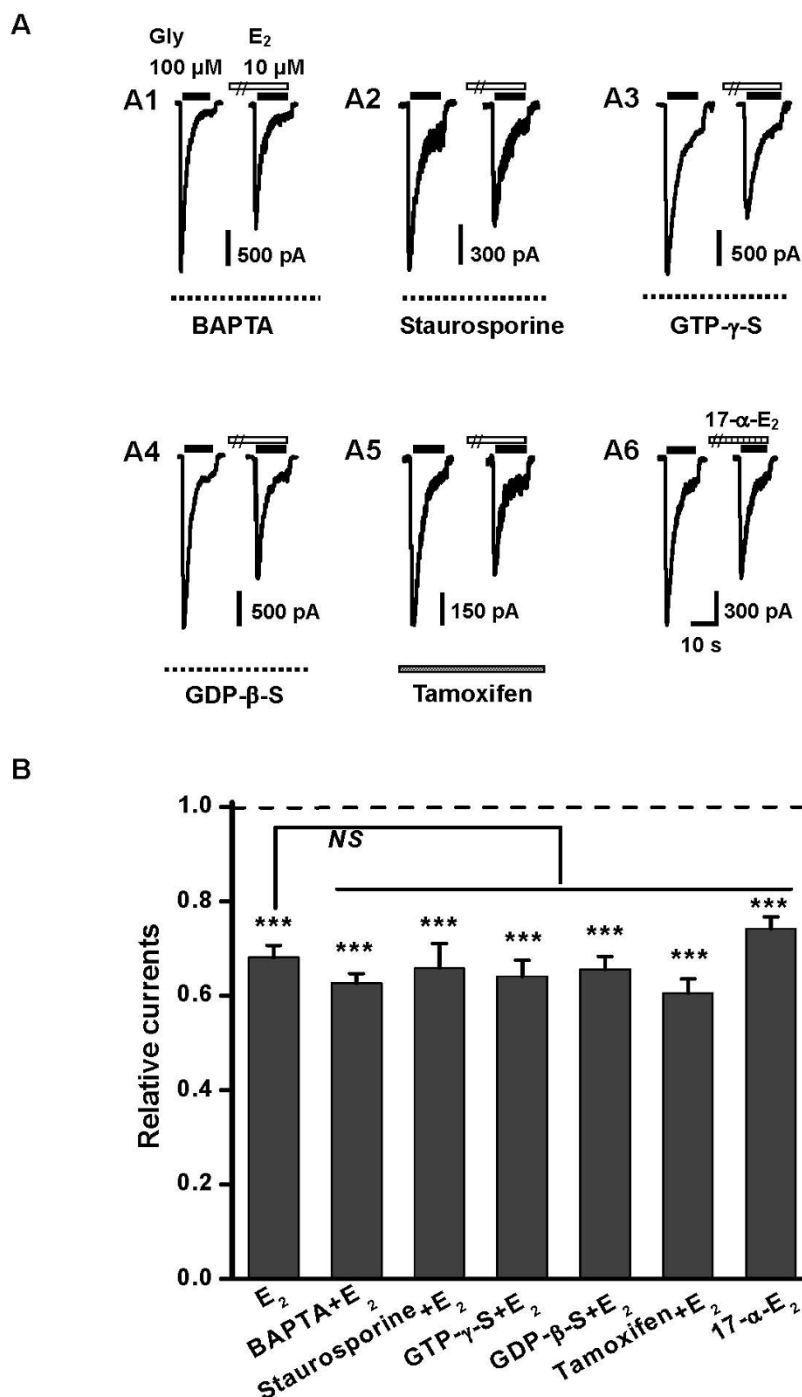
Figure 2
E_2-induced inhibition of I_{Gly} is independent of intracellular signaling pathways and classical estrogen receptors (ERs). (A) Sample traces illustrating the inhibitory effects of E_2 on the peak I_{Gly} under the conditions of intracellular application 15 mM BAPTA (A1), 5 μM staurosporine (A2), 0.5 mM GTP-γ-S (A3) and 0.5 mM GTP-β-S (A4), respectively. A5, Effect of E_2 on I_{Gly} after incubation of neurons with tamoxifen for 2 h. A6, Effect of 17-α-E_2 on I_{Gly}. (B) Pooled data summarizing the effect of E_2 on I_{Gly} under various conditions shown in *A*. Each column represents the average values from 4–6 neurons, ***$P < 0.001$, Paired Student's *t*-test, compared with control without adding E_2 or 17-α-E_2 (dashed line). *NS* indicates no significant difference in this and the following figures.
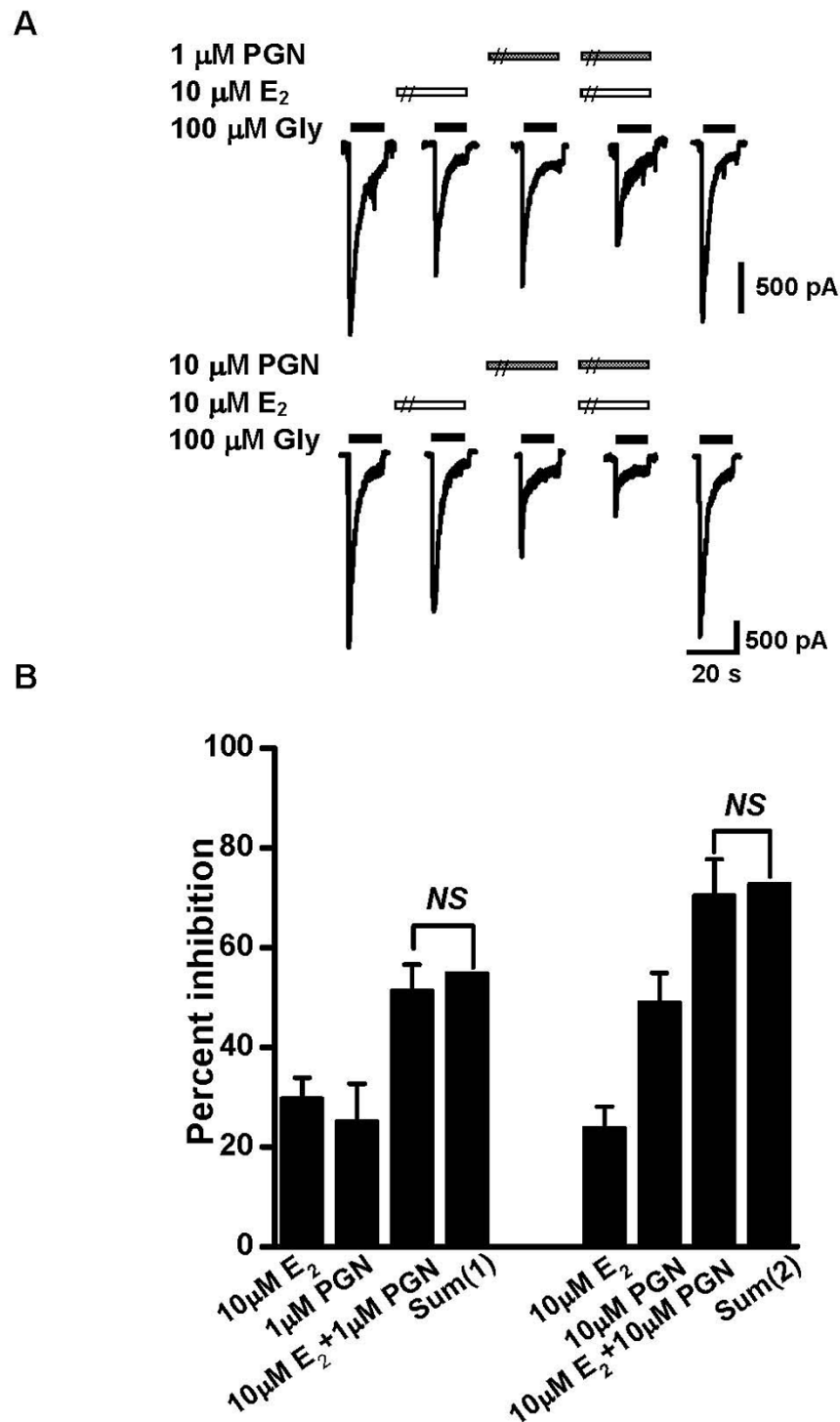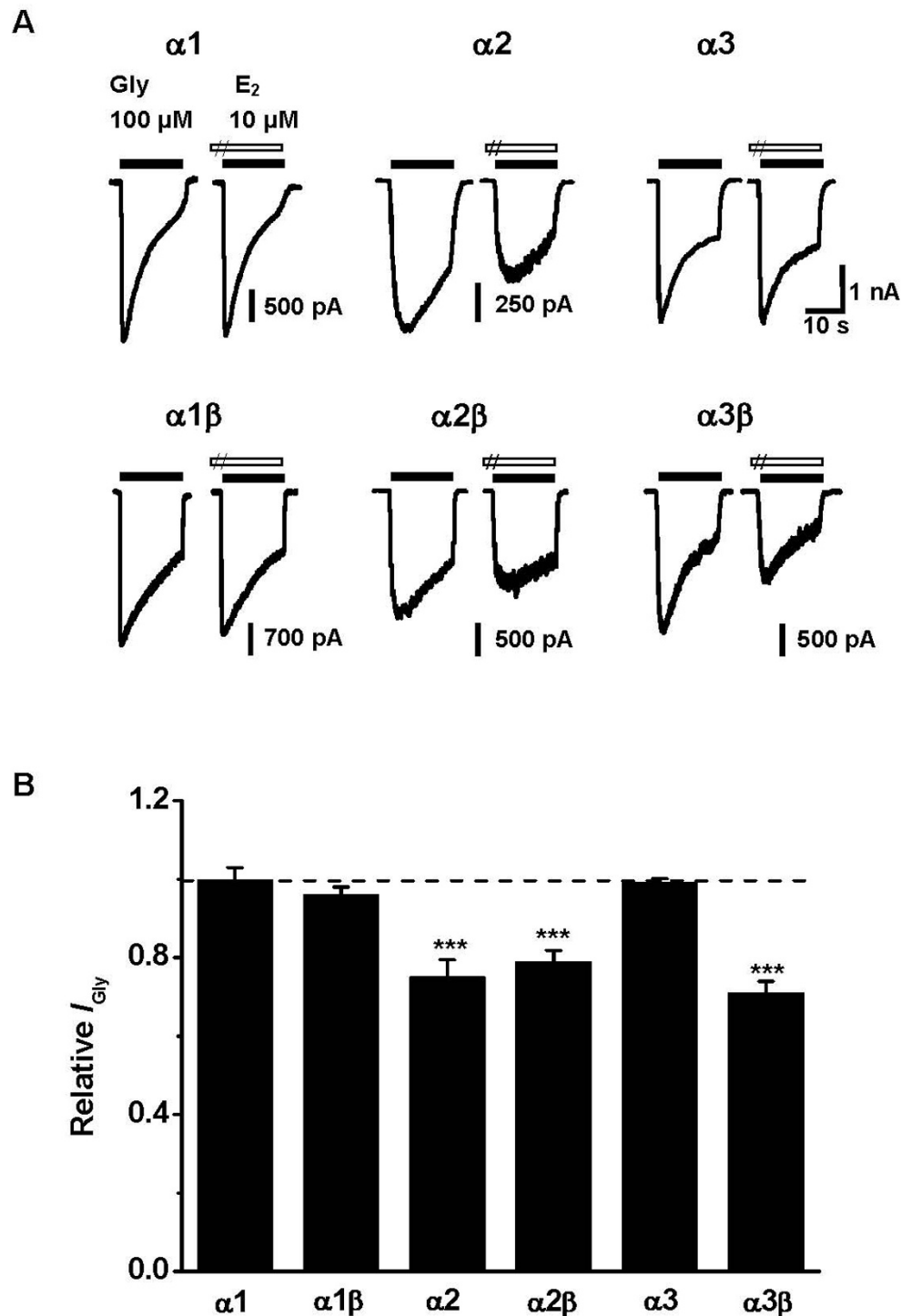
Figure 3
Interactions of E$_2$ and PGN on I_{Gly}. (A) Sample traces illustrating the additive effect of E$_2$ and PGN on I_{Gly}. (B) Summary of results from all experiments similar to those shown in *A* (n = 4–5). Sum (1) is the expected linear summation of the inhibition induced by 10 μM E$_2$ and 1 μM PGN; Sum (2) is the expected linear summation of the inhibition induced by 10 μM E$_2$ and 10 μM PGN. *P* > 0.05, Unpaired Student's *t*-test.

Figure 4
Inhibitory effect of E$_2$ on recombinant GlyRs. (A) Sample traces demonstrating the effects of 10 μM E$_2$ on various homo-meric and heteromeric GlyRs. (B) Summary of results from all experiments similar to those shown in *A*. E$_2$ selectively inhibited the peak amplitude of I_{Gly} mediated by α2-containing GlyRs and α3β heteromeric GlyR. Each column represents the average value of 6–11 neurons. ***$P < 0.001$, Paired Student's *t*-test, compared with the control without E$_2$ treatment (dashed line).
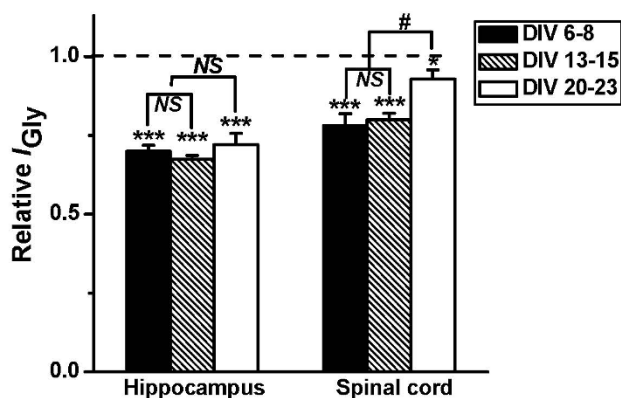
Figure 5
Developmental regulation of E$_2$ inhibition on I_{Gly}. Hippocampal and spinal cord neurons in culture were used for electrophysiological recording following 6–8, 13–15 and 20–23 days of *in vitro* (DIV) differentiation to examine the developmental dependence of E$_2$ inhibition on I_{Gly}. Each column represents the average value of 5–8 neurons. * $P < 0.05$ and *** $P < 0.001$ (Student's paired *t* test, n = 8), compared to control without adding E$_2$; # $P < 0.05$ (one-way ANOVA, n = 8), comparing DIV 20–23 with either DIV 6–8 or DIV 13–15.

nal fluid has been estimated to be in the micromolar range [40]. In order to magnify the current, we recorded the GlyR-mediated tonic current by adding 20 μM glycine to ACSF in addition to the presence of GlyT1 inhibitor sarcosine (0.5 mM) [25]. At the same time, TTX (0.3 μM) was added in the bath to reduce random baseline current fluctuations, and 10 μM bicuculline, 10 μM DL-2-amino-5-phosphovaleric acid (APV) and 3 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were added to block GABA$_A$ and ionotropic glutamate receptors, respectively. After recording a control period in the cocktail solution, E$_2$ at 10 μM was superfused (Figure 6A). As shown in Figure 6B, the GlyR-mediated tonic current was significantly reduced to 69.3 ± 5.8% of control by E$_2$ perfusion ($P = 0.043$, n = 8, Wilcoxon matched-pairs signed-ranks test), a level compatible with that obtained in the cultured neurons (Figure 1B) or HEK 293 cells expressing recombinant α2- and α2β-GlyRs (Figure 4B).

Discussion

Here, we present evidence that in cultured HIP and SDH neurons, E$_2$ directly inhibited GlyRs which are likely composed of α2 or α3 subunit. Furthermore, the GlyR-mediated tonic current in HIP slices was reduced by E$_2$. Thus, in addition to the well-known signaling pathways of E$_2$ via ERs, the GlyR may be an additional central target of E$_2$. Since GlyRs are important regulators of spinal nociception [26,41,42], and affect HIP network activity [19,20],

our findings may shed new light on the gender differences in pain sensation at the CNS level.

Mechanisms underlying E$_2$ inhibition of GlyRs

Estrogen exerts effects in the CNS primarily by activating ERs [3]. In this study, however, several lines of evidence suggest that E$_2$ inhibited I_{Gly} through a direct interaction with plasma membrane GlyRs. First, the effects of E$_2$ are most likely non-genomic as the inhibition occurred within seconds following E$_2$ application and persisted in large outside-out patches devoid of nuclei. Second, it is unlikely that E$_2$ physically disrupts the plasma membrane via a nonspecific way as E$_2$ only affected α2 subunit-containing and heteromeric α3β-GlyRs. Moreover, when E$_2$ was applied, the membrane capacitance remained unchanged, indicating that the membrane structure was unaltered (see Materials and Methods). Third, E$_2$ inhibitory effect persisted in the presence of tamoxifen, the antagonist of classical ERs [43], or 17-α-E$_2$, the inactive stereoisomer of E$_2$ [44]. In addition, E$_2$ inhibited the recombinant GlyRs expressed in HEK293 cells which are devoid of classical ERs [45]. Finally, in the presence of the calcium chelator, the protein kinase inhibitor or G-protein modulators, the E$_2$-induced inhibition remained unchanged. These results strongly support the notion that E$_2$ inhibits GlyRs independent of any intracellular signaling pathways activated by either classical ERs or novel ERs [9-11].

Our data suggest that E$_2$ inhibited I_{Gly} in a noncompetitive manner because E$_2$ reduced I_{Gly} independent of glycine concentrations, and the effect can be additive with that of PGN, a competitive steroid inhibitor of GlyR without changing the membrane viscosity [39,46]. Two general mechanisms may underlie the noncompetitive inhibition of E$_2$ on GlyR, open-channel blocker or allosteric modulation. Nevertheless, E$_2$ is not charged at physiological pH and inhibited I_{Gly} independent of membrane potentials (P.J. and T.L.X., unpublished data), which makes it unlikely that E$_2$ acts as an open-channel blocker. Thus, we propose that E$_2$ directly binds to and allosterically inhibits GlyRs.

Functional implications

Generally, the physiological concentration of estradiol in plasma is in the nanomolar range [6]. In the present study, the concentrations at which E$_2$ exerted significant effects on GlyRs were much higher than its physiological level. However, in the CNS of both male and female rats, estrogen can be *de novo* synthesized and accumulated locally [30,47], leading to E$_2$ concentration in the cerebrospinal fluid far surpass those measured in plasma. For example, a previous study has demonstrated that the concentration of estradiol in the hippocampus of male rats is six-fold higher than that in plasma [48]. More interest-
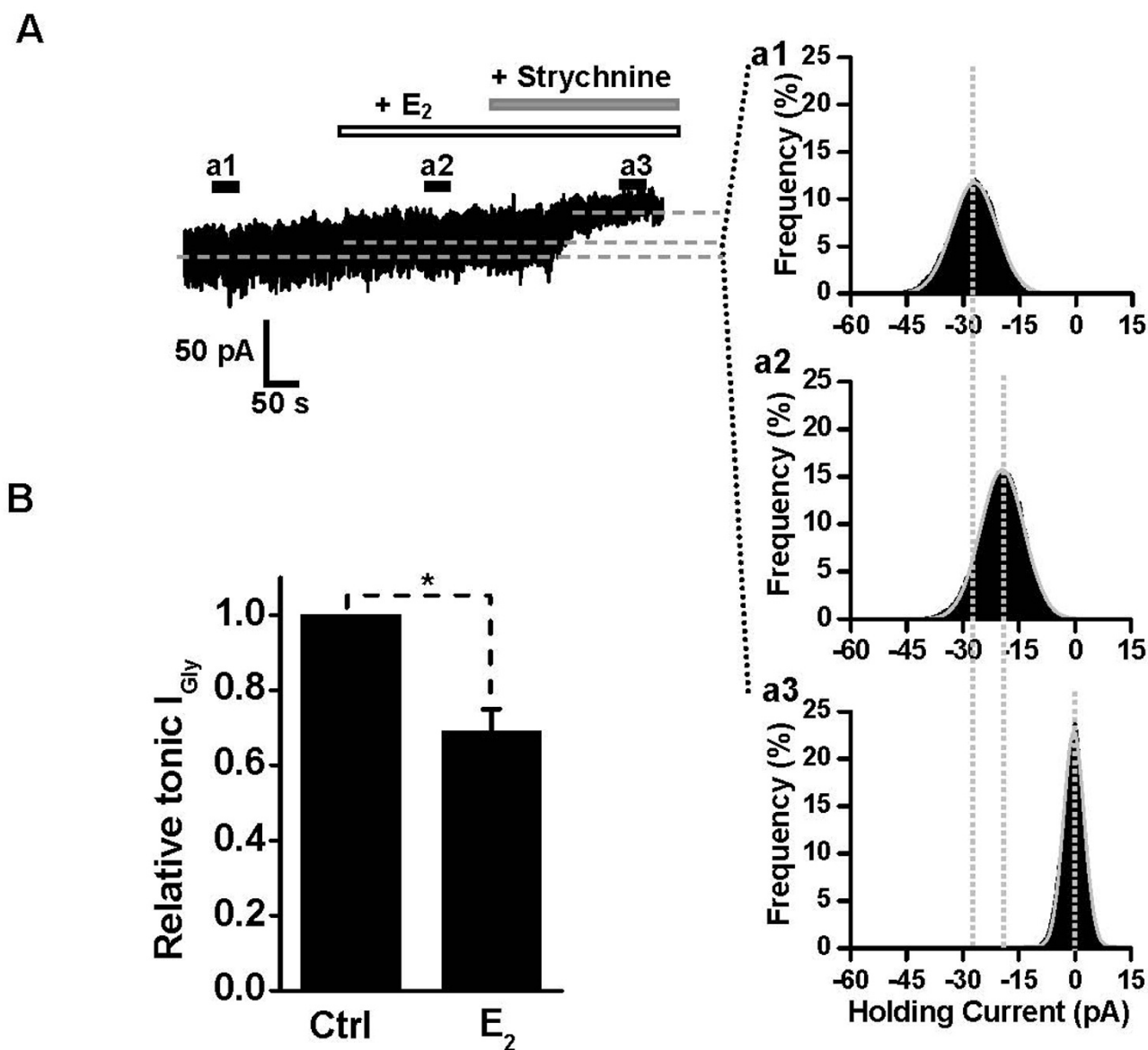
Figure 6
Inhibitory effect of E$_2$ on GlyR-mediated tonic current in HIP slices. (A) Left, Whole-cell voltage-clamp recording showing the current in the presence of 0.3 μM TTX, 10 μM bicuculline, 3 μM CNQX, 10 μM APV, 20 μM glycine and 0.5 mM sarcosine. Application of strychnine (2 μM) decreased the membrane current noise and revealed the tonically activated GlyR current. In the presence of E$_2$ (10 μM), the amplitude of GlyR-mediated tonic current was significantly reduced. Right (a1–a3), Gaussian fit to all-point histograms of 30 s traces at the time point a1, a2 and a3 (A, inset). The differences among the Gaussian means are marked by the dotted lines. (B) The normalized GlyR-mediated tonic current in the absence or presence of E$_2$ (n = 8). *P < 0.05, Paired Student's *t*-test, compared with the control without E$_2$ treatment.

ingly, a recent study indicates that forebrain estradiol levels in zebra finches are also acutely increased during social interactions [49]. In addition, certain cofactors, like sexual hormone-binding proteins, can make E$_2$ to achieve an effect even at low concentrations [50]. Therefore, it is reasonable to speculate that, under certain physiological or

pathological condition, locally elevated endogenous estrogen may have an impact on GlyRs through the process revealed in this study.

In HEK293 cells expressing recombinant GlyRs, we found that E$_2$ significantly inhibited α2-GlyRs. Accumulative evi-

dence demonstrates that the α2-GlyRs distribute throughout the developing CNS and in the mature hippocampus [21,51,52]. In CA1 region of HIP slices, we observed that GlyR-mediated tonic currents, which are likely mediated by α2-GlyRs [51-53], were reduced to a level compatible with that measured in cultured neurons or HEK 293 cells expressing recombinant GlyRs. The tonic activation of the α2 subunit-containing GlyRs is functionally important in the development of the cortex [54], the retina [28] and the spinal cord [29]. Furthermore, previous studies have shown that GlyR subunit composition of spinal cord neurons changes during development from α2 to α1 predominance both in cultures and in *in vivo* conditions [27,55]. Because of this switch of GlyR subunit composition in the spinal cord during development, a previous study [56] suggested that the selective inhibition of progesterone on α2-GlyRs endows an important role of progesterone in the developing spinal cord. Since the levels of estrogen fluctuate in both males and females during brain development [31], E_2-induced inhibition of GlyRs may contribute to the modulation of estrogen on the early CNS development. It remains unexplored whether GlyRs also participate in regulation of neuronal excitability, through E_2-induced disinhibition in the mature hippocampus [14].

Under certain disease conditions, for example, premenstrual dysphoric disorder (PMDD), anxiety is enhanced when neuronal excitability is disturbed during the ovarian cycle [57]. A recent study reported that the periodic alterations of the $GABA_A$R-mediated tonic inhibition, which resulted from the fluctuation of hormones in the hippocampus during estrous cycle, play a crucial role in altered seizure susceptibility and anxiety during PMDD [58]. Similar to the tonic GABAergic inhibition, GlyRs-mediated tonic currents modulate neuronal excitation and network function [18,19,24,25]. Our present data showed that E_2 can inhibit more than 30% of the tonic I_{Gly} in hippocampal slices. Therefore, the significant inhibition of I_{Gly} produced by E_2 may have strong effect on the neuronal excitability, suggesting an alternative cellular mechanism by which periodic sex hormone fluctuations affect CNS neuronal excitation and cause mood disorders. Considering the ubiquitous distribution profiles of $GABA_A$Rs in the CNS and the hypnotic side-effect of GABA-mimic drugs, the present finding suggests new directions for treatment of PMDD by using GlyR-enhancing drugs and/or glycine reuptake inhibitors [25].

Besides, sex-related difference in pain perception has been revealed by previous studies which indicate that noxious stimuli are perceived as more painful by women than by men [13,43]. Although sex hormones have been shown to be involved in this difference, the underlying mechanisms are still unknown. Functional α3β-GlyRs exist in inhibitory synapse of SDH neurons, which is the first site for integration, relay and modulation of nociceptive information from nociceptor [59]. Moreover, previous studies have suggested that α3β-GlyRs in the SDH neurons regulate inflammatory pain sensitization [26,41]. In this study, we noted that E_2 significantly inhibited I_{Gly} in cultured SDH neurons and the current mediated by α3β-GlyRs expressed in HEK293 cells, providing important insights into the mechanisms underlying gender differences in pain sensation at the CNS level.

Finally, several studies indicate that the expression of high affinity GlyR subunit α2^{192L} and α3^{185L} produced by RNA editing [60] was increased in the brain after experimentally induced brain lesion of rat [61] and in temporal lobe epilepsy patient with a severe course of disease [62]. According to the selectivity of E_2 inhibition on α2 and α3-containing GlyR, E_2 thus may play a role through modulating these high affinity GlyRs in some pathological situations such as temporal lobe epilepsy and brain damage.

Conclusion
We demonstrated that the neuronal GlyR is a novel molecular target of E_2 which directly inhibits the function of GlyRs in the HIP and SDH regions. Through their impact on GlyRs in the CNS, sex hormones may regulate neuronal excitability and contribute to premenstrual dysphoric disorder and sex-related pain sensation under both physiological and pathological conditions.

Methods
The care and use of animals in these experiments followed the guidelines of the Institutional Animals Care and Use Committee of the Institute of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. All efforts were made to minimize the number of animals used and their suffering.

Cell culture
Cultures of HIP neurons were prepared as previously described [63] with some modifications. Briefly, hippocampus from neonatal (< 24-hour-old) Sprague-Dawley rats with identified sex were dissociated in Ca^{2+}-free saline with sucrose (20 mM) and hippocampal neurons were isolated using a standard enzyme treatment protocol. Cultures of SDH neurons were prepared from embryonic day 15 (E15) Sprague-Dawley rats as previously described [39]. The neurons were plated (1–5 × 10^5 cell/ml) on poly-L-lysine (Sigma, USA) coated cover glasses and grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) with L-glutamine plus 10% fetal bovine serum (Gibco). The neurons were allowed to attach the cover glasses for 24 h, after which the medium was changed to neuron-basal medium (1.5 ml, Gibco) with 2% B27 (Gibco) and replaced every 3–4 days. Treatment with 5-fluoro-5'-deoxyuridine (20 μg/ml, Sigma, St.

Louis, MO) on the fourth day after plating was used to block cell division of non-neuronal cells, which helped to stabilize the cell population. The cultures were maintained at 37°C in a 5% CO_2 humidified atmosphere. Cells were used for electrophysiological recordings 7–23 days after plating.

Slice preparation
Experiments were performed on 400 μm transverse hippocampal slices from 14- to 17-day-old Sprague-Dawley rats. After decapitation, the brain was removed and placed in oxygenated (95% O_2/5% CO_2) artificial cerebrospinal fluid (ACSF) at 4°C. Slices were cut from the dorsal hippocampus with a vibratome (Leika VT 1000S) and maintained at room temperature (23–25°C) in a holding chamber filled with oxygenated ACSF. After an equilibration period of at least 2 h, a single slice was transferred to the recording chamber, where it was continuously perfused with oxygenated ACSF (23–25°C) at a flow rate of 2.5–3 ml/min.

Expression of recombinant GlyRs
All constructs were expressed in HEK293 cells as previously reported [39]. In brief, HEK293 cells were cultured at 37°C in a humidified atmosphere of 5% CO2 and 95% air. Cells were maintained in DMEM supplemented with 2 mM L-glutamine, 10% fetal bovine serum, and 100 units/ml penicillin/streptomycin (all from Invitrogen). Transient transfection of HEK293 cells was carried out by using the Lipofectamine 2000 reagent (Invitrogen) according to the supplied protocol. Co-transfection with a green fluorescent protein expression vector, pEGFP-N1, was used to enable identification of transfected cells for patch clamping in some experiments. When co-transfecting the GlyR α and β subunits, their respective cDNAs were combined in a ratio of 1:2 to ensure the formation of functional heterooligomers. Taking advantage of the insensitivity of αβ heteromeric GlyR to picrotoxin [64], we further tested the picrotoxin sensitivity to ensure the formation of heterooligomers. After exposure to transfection solution for 6 h, cells were washed twice using the culture medium and used for electrophysiological recordings over following 16–48 h.

Solutions and drugs
The standard external solution for cultured neurons recording contained (mM): 150 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 N-hydroxyethylpiperazine-NV-2-ethanesulphonic acid (HEPES), 10 Glucose (pH 7.4; osmolarity 310–320 mOsm/l). The patch pipette solution for cultured neurons recording was (mM): 120 KCl, 30 NaCl, 1 $MgCl_2$, 0.5 $CaCl_2$, 5 EGTA, 2 Mg-ATP, 10 HEPES. The internal solution was adjusted to pH 7.2 with Tris-base. The ACSF for slices incubation and recording was composed of (mM): 126 NaCl, 2.5 KCl, 2 $MgSO_4$, 1.25

NaH_2PO_4, 26 $NaHCO_3$, 2 $CaCl_2$ and 10 D-glucose, aerated with 95% O_2 and 5% CO_2 at a final pH of 7.4. The osmolarity of the ACSF was 289–295 mOsm/l. The ionic composition of the internal solution for slice recording was (mM): 140 CsCl, 1 $MgCl_2$, 10 HEPES, 0.1 EGTA, 4 NaCl, 2 MgATP and 0.3 NaGTP, adjusted to pH 7.2. Drugs used in the present experiments were purchased from Sigma. Steroidal agents were initially dissolved as concentrated stock solutions in dimethylsulphoxide (DMSO) and subsequently diluted to the desired concentration in standard external solution. The final concentration of DMSO employed in the experiments was always ≤ 0.1%, which had no detectable effect on I_{Gly} in vehicle control experiments. Other drugs were first dissolved in ion-free water and then diluted to the final concentrations in the standard external solution or ACSF just prior to use. Unless otherwise indicated, drugs were applied using a rapid application technique termed the 'Y-tube' method throughout the experiments. This system allows a complete exchange of external solution surrounding a neuron within 20 ms [65].

Electrophysiological recordings and data analysis
Conventional whole-cell patch-clamp recording was performed under voltage-clamp conditions. Patch pipettes were pulled from glass capillaries with an outer diameter of 1.5 mm on a two-stage puller (PP-830, Narishige, Tokyo, Japan). The resistance between the recording electrode filled with pipette solution and the reference electrode was 4–6 MΩ. Membrane currents were measured using a patch-clamp amplifier (Axon 200B, Axon Instruments, Foster City, CA, USA), sampled and analyzed using a Digidata 1320A interface and a personal computer with Clampex and Clampfit software (Version 9.0.1, Axon Instruments). In most experiments, 70–90% series resistance was compensated. To make outside-out patch, after obtaining the whole-cell recording configuration, the patch was excised by carefully withdrawing the patch pipette from the cell [66]. Additionally, in some experiments that drugs were applied in pipette, the current was measured at least 5 minutes after whole-cell configuration was established to ensure cell dialysis [67]. Unless otherwise indicated, the membrane potential was held at -50 mV throughout the experiment. All experiments were carried out at room temperature (22–25°C).

Membrane capacitance measurements
Whole-cell voltage clamp was used to step membrane potential from -70 to +20 mV. A cesium based pipette solution was used to block voltage-gated potassium channels. The capacitance transient was recorded in HEK293 cell expressing α2-GlyRs in the absence or presence of 10 μM E_2. According to the calculation method of capacitance provided by the previous study [46], the capacitance of HEK293 cell expressing α2-GlyRs was estimated to be

33.3 ± 2.2 pF and 33.2 ± 2.4 pF in the absence or presence of E_2, respectively. The membrane capacitance was not changed by E_2, indicating that the alteration of plasma membrane capacitance was not involved in E_2 inhibition of GlyRs.

Data analysis

Clampfit software was used for data analysis. The continuous theoretical curves for concentration-response relationship of glycine in the presence or absence of steroids were drawn according to a modified Michaelis-Menten equation by the method of least-squares (the Newton-Raphson method) after normalizing the amplitude of the response:

$$I = I_{max} \, C^h/(C^h + EC_{50}{}^h)$$

where I is the normalized value of the current, I_{max} the maximal response, C the drug concentration, EC_{50} the concentration which induced the half-maximal response and h the apparent Hill coefficient. The curve for the effect of E_2 on I_{Gly} was fitted using the following equation:

$$I = I_{max} \, (IC_{50})^h/(C^h + IC_{50}{}^h)$$

where IC_{50} represents the concentration that induced the half-maximal inhibitory effect.

For analysis of the GlyR-mediated tonic currents in HIP slices, all-point histograms of 30 s epochs at period a1, a2 and a3 was used to measure the baseline current in different condition of drug application (see Figure 6A), and a Gaussian distribution was fitted to the histogram at period a1, a2 and a3. The GlyR-mediated tonic current was revealed by strychnine application. Therefore, the difference between the means of the fitted Gaussians at period a1 and a3 represents the GlyR-mediated tonic current in the absence of E_2. Similarly, the difference between period a2 and a3 represents the GlyR-mediated tonic current in the presence of E_2.

The statistical comparisons were carried out by using Student's *t*-test for two groups' comparison, and one-way analysis of variance (ANOVA) for multiple comparisons. All data were reported as the mean ± standard error (S.E.M.). *P* and *n* represent the value of significance and the number of neurons, respectively. Statistically significant differences were assumed as *P* < 0.05.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PJ and YK carried out all electrophysiological experiments and wrote the manuscript. XBZ participated in the electro-

physiological experiment in slices and revised the manuscript. WW participated in cell culture. TLX and CFL conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.

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