RESEARCH PAPER

Modulation of presynaptic GABAA receptors by endogenous neurosteroids

B-G Kim[1], J-H Cho[1], I-S Choi[1], M-G Lee[2,3] and I-S Jang[1,3]

[1]Department of Pharmacology, School of Dentistry, Kyungpook National University, Daegu, Korea, [2]Department of Pharmacology, School of Medicine, Kyungpook National University, Daegu, Korea, and [3]Brain Science & Engineering Institute, Kyungpook National University, Daegu, Korea

BACKGROUND AND PURPOSE

Although 3α-hydroxy, 5α-reduced pregnane steroids, such as allopregnanolone (AlloP) and tetrahydrodeoxycorticosterone, are endogenous positive modulators of postsynaptic GABAA receptors, the functional roles of endogenous neurosteroids in synaptic transmission are still largely unknown.

EXPERIMENTAL APPROACH

In this study, the effect of AlloP on spontaneous glutamate release was examined in mechanically isolated dentate gyrus hilar neurons by use of the conventional whole-cell patch-clamp technique.

KEY RESULTS

AlloP increased the frequency of glutamatergic spontaneous excitatory postsynaptic currents (sEPSCs) in a dose-dependent manner. The AlloP-induced increase in sEPSC frequency was completely blocked by a non-competitive GABAA receptor blocker, tetrodotoxin or Cd^{2+}, suggesting that AlloP acts on presynaptic GABAA receptors to depolarize presynaptic nerve terminals to increase the probability of spontaneous glutamate release. On the other hand, γ-cyclodextrin (γ-CD) significantly decreased the basal frequency of sEPSCs. However, γ-CD failed to decrease the basal frequency of sEPSCs in the presence of a non-competitive GABAA receptor antagonist or tetrodotoxin. In addition, γ-CD failed to decrease the basal frequency of sEPSCs after blocking the synthesis of endogenous 5α-reduced pregnane steroids. Furthermore, γ-CD decreased the extent of muscimol-induced increase in sEPSC frequency, suggesting that endogenous neurosteroids can directly activate and/or potentiate presynaptic GABAA receptors to affect spontaneous glutamate release onto hilar neurons.

CONCLUSIONS AND IMPLICATIONS

The modulation of presynaptic GABAA receptors by endogenous neurosteroids might affect the excitability of the dentate gyrus-hilus-CA3 network, and thus contribute, at least in part, to some pathological conditions, such as catamenial epilepsy and premenstrual dysphoric disorder.

Abbreviations

3α-HSD, 3α-hydroxysteroid dehydrogenase; γ-CD, γ-cyclodextrin; AlloP, allopregnanolone; APV, DL-2-amino-5-phosphonovaleric acid; $[Cl^-]_i$, intracellular Cl^- concentration; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DG, dentate gyrus; eEPSCs, action potential-dependent EPSCs; KA, kainic acid; K-S, Kolmogorov–Smirnov; NKCC1, Na-K-Cl cotransporter type 1; PPR, paired-pulse ratio; sEPSCs, spontaneous EPSCs; SR95531, 6-imino-3-(4-methoxyphenyl)-1(6H)-pyridazinebutanoic acid HBr; THDOC, tetrahydrodeoxycorticosterone; TTX, tetrodotoxin; VDCCs, voltage-dependent Ca^{2+} channels; VDNCs, voltage-dependent Na^+ channels

Introduction

3α-Hydroxy, 5α-reduced pregnane steroids, such as allopregnanolone (AlloP) and tetrahydrodeoxycorticosterone (THDOC), are *de novo* synthesized by 5α-reductase and 3α-hydroxysteroid dehydrogenase (3α-HSD) from progesterone and deoxycorticosterone, respectively (Mellon and Griffin, 2002), and are widely distributed in the brain including the hippocampus and cortex (Saalmann *et al.*, 2007). These neurosteroids have inhibitory actions against neuronal excitability, thus exhibiting anxiolytic, sedative and anticonvulsant effects (Wieland *et al.*, 1991; Kokate *et al.*, 1994). These inhibitory actions would mainly be mediated by the positive modulation of GABA$_A$ receptors, as AlloP, at nanomolar concentrations, potentiated the GABA$_A$ receptor-mediated currents (Zhu and Vicini, 1997; Stell *et al.*, 2003; Akk *et al.*, 2005). At somewhat higher concentrations ($\geq$30 nM), however, these neurosteroids directly open GABA$_A$ receptors in the absence of ligand (e.g. GABA) binding (Majewska *et al.*, 1986; Hosie *et al.*, 2006). The levels of 3α-hydroxy, 5α-reduced pregnane steroids might be locally regulated by *de novo* synthesis and/or generated from circulating progesterone within the brain Moreover, the neurosteroids can reach concentrations approaching 100 nM during the oestrous cycle as well as during acute stress (Purdy *et al.*, 1991; Corpéchot *et al.*, 1993). Therefore, changes in the levels of endogenous 3α-hydroxy, 5α-reduced pregnane steroids might be closely related to premenstrual and post-partum dysphoric disorders (Follesa *et al.*, 2004), depression (Uzunova *et al.*, 1998), ethanol withdrawal (Gililland-Kaufman *et al.*, 2008) and catamenial epilepsy (Reddy and Rogawski, 2000).

Growing evidence has shown that GABA$_A$ receptors are also expressed on presynaptic terminals in a variety of brain regions, and that their activation induces presynaptic depolarization in order to modulate neurotransmitter release from nerve terminals (Eccles *et al.*, 1963; Segev, 1990; Cattaert and El Manira, 1999; Jang *et al.*, 2001; 2002; Turecek and Trussell, 2002), including hippocampal mossy fibres (Ruiz *et al.*, 2003; Jang *et al.*, 2006; Nakamura *et al.*, 2007). Presynaptic GABA$_A$ receptors can be targets for some pharmacological agents (Han *et al.*, 2009), as GABA$_A$ receptors have multiple allosteric binding sites for benzodiazepines, general anaesthetics, synthetic and endogenous neurosteroids, alcohols and divalent metal ions (Barnard *et al.*, 1998; Belelli *et al.*, 1999; Mohler *et al.*, 2002; Whiting, 2003). For example, a recent study has shown that exogenously applied 3α-hydroxy, 5α-reduced pregnane steroids enhances action potential-dependent Ca^{2+} transients and facilitates glutamatergic transmission to CA3 pyramidal neurons (Ruiz *et al.*, 2010). In addition, our previous studies have shown that presynaptic GABA$_A$ receptors expressed on hippocampal glutamatergic nerve terminals are highly sensitive to muscimol, a GABA$_A$ receptor agonist (Jang *et al.*, 2006; Han *et al.*, 2009). Given that mRNAs of both 5α-reductase and 3α-HSD are exclusively expressed on hippocampal glutamatergic neurons (Agís-Balboa *et al.*, 2006), and that 3α-hydroxy, 5α-reduced pregnane steroids, even at low nanomolar concentrations, potentiate GABA$_A$ receptors, the higher sensitivity of presynaptic GABA$_A$ receptors to agonists might be due to the intrinsic modulation of GABA$_A$ receptors by endogenous 3α-hydroxy, 5α-reduced pregnane steroids. In the present study, therefore, we have investigated the functional roles of AlloP and endogenous neurosteroids in glutamatergic transmission in acutely isolated dentate gyrus (DG) hilar neurons.

Methods

Preparation of neurons

All animal care and experimental procedures complied with the guidelines for the care and use of animals approved by the Council of the Physiological Society of Korea and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize both the number of animals used and their suffering.

Sprague Dawley rats (12- to 16-day-old), except where indicated, were decapitated under pentobarbital anaesthesia (50 mg·kg^{-1}, i.p.). The brain was dissected and transversely sliced at a thickness of 400 μm using a microslicer (VT1000S; Leica, Nussloch, Germany). Slices containing the hippocampus were kept in an incubation medium (or bath solution, see Solutions) saturated with 95% O$_2$ and 5% CO$_2$ at room temperature (22–24°C) for at least 1 h before the mechanical dissociation. For dissociation, slices were transferred into a 35 mm culture dish (Primaria 3801; Becton Dickinson, Rutherford, NJ, USA) containing a standard external solution (see below), and the hilar region of the hippocampus was identified under a binocular microscope (SMZ-1; Nikon, Tokyo, Japan). Details of the mechanical dissociation have been described previously (Rhee *et al.*, 1999). Briefly, mechanical dissociation was accomplished using a custom-built vibration device and a fire-polished glass pipette oscillating at about 50–60 Hz (0.3–0.5 mm) on the surface of the hilar region, except where indicated. The slices were removed and the mechanically dissociated neurons were allowed to settle and adhere to the bottom of the dish for 15 min. These dissociated neurons lost the most distal processes but retained a short portion (~50 μm in a length) of their proximal dendrites (Lee *et al.*, 2009).

In a subset of experiments, the effect of AlloP on glutamate release was tested using the slice preparation. For the slice preparation, the hippocampus was transversely sliced at a thickness of 300 μm by use of a microslicer. Slices containing the hippocampus were kept in the bath solution (see below) saturated with 95% O$_2$ and 5% CO$_2$ at room temperature (22–24°C) for at least 1 h. Thereafter a slice containing the hippocampus was transferred into a recording chamber, and the hilar region was identified under an upright microscope (E600FN, Nikon, Tokyo, Japan) with a water-immersion objective (X 40). The bath solution was perfused at 2 mL·min^{-1} using a peristaltic pump (MP-1000, EYELA, Tokyo, Japan).

Electrophysiological recordings

All electrophysiological recordings were performed using conventional whole-cell patch recordings and a standard patch-clamp amplifier (Axopatch 200B; Molecular Devices, Union City, CA, USA). Neurons were voltage-clamped at a holding potential (V_H) of –60 mV. Patch pipettes were made from borosilicate capillary glass (1.5 mm outer diameter,

0.9 mm inner diameter; G–1.5; Narishige, Tokyo, Japan) by use of a pipette puller (P-97; Sutter Instrument Co., Novato, CA, USA). The resistance of the recording pipettes filled with an internal (pipette) solution was 4–6 MΩ. The liquid junction potential (~–11 mV, measured by exchanging bath solution from internal solution to standard external solution) and pipette capacitance were compensated for. Neurons were viewed under phase contrast on an inverted microscope (TE-2000; Nikon, Tokyo, Japan). Membrane currents were filtered at 2 kHz (Axopatch 200B), digitized at 10 kHz, and stored on a computer equipped with pCLAMP 10 (Molecular Devices). When recording, 10 mV hyperpolarizing step pulses (30 ms in duration) were periodically delivered to monitor the access resistance. All experiments were performed at room temperature (22–24°C).

In the slice preparation, electrical measurements were performed using conventional whole-cell patch recordings with a patch-clamp amplifier (MultiClamp 700B; Molecular Devices). To record action potential-dependent EPSCs (eEPSCs), a glass stimulation pipette (~10 μm diameter), filled with a bath solution, was positioned around the granule cell layer. Brief paired pulses (500 μs, 100–200 μA, 10 Hz) were applied by the pipette at a stimulation frequency of 0.05 Hz using a stimulator (SEN-7203, Nihon Kohden, Tokyo, Japan) equipped with an isolator unit (SS-701J, Nihon Kohden). Data were filtered at 2 kHz and digitized at 10 kHz.

Data analysis

The amplitude of eEPSCs was calculated by subtracting the baseline from the respective peak amplitude. Spontaneous EPSCs (sEPSCs) were counted and analysed using the Mini-Analysis program (Synaptosoft, Inc., Decatur, GA, USA), as described previously (Jang et al., 2002; 2006). Briefly, sEPSCs were screened automatically using an amplitude threshold of 10 pA and then visually accepted or rejected based upon the rise and decay times. The average values of both the frequency and amplitude of sEPSCs during the control period (5–10 min) were calculated for each recording, and the frequency and amplitude of all the events during the AlloP or muscimol application (1–2 min) were normalized to these values. The effects of AlloP or muscimol were quantified as a percentage increase in sEPSC frequency compared with the control values. The inter-event intervals and amplitudes of a large number of synaptic events obtained from the same neuron were examined by constructing cumulative probability distributions and compared using the Kolmogorov–Smirnov (K-S) test with Stat View software (SAS Institute, Inc., Cary, NC, USA). Numerical values are provided as the mean ± SEM using values normalized to the control. Significant differences in the mean amplitude and frequency were tested using a Student's paired two-tailed t-test with absolute values rather than normalized ones. Values of $P < 0.05$ were considered significant.

Solutions

The ionic composition of the incubation medium consisted of (in mM) 124 NaCl, 3 KCl, 1.5 KH$_2$PO$_4$, 24 NaHCO$_3$, 2 CaCl$_2$, 1.3 MgSO$_4$ and 10 glucose saturated with 95% O$_2$ and 5% CO$_2$. The pH was about 7.45. The standard external solution was (in mM) 150 NaCl, 3 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 glucose

and 10 HEPES, and was adjusted to a pH of 7.4 with Tris-base. For recording sEPSCs, these standard external or bath solutions routinely contained 50 μM DL-2-amino-5-phosphonovaleric acid (APV) to block NMDA receptors. The ionic composition of the internal solution consisted of (in mM) 135 CsF, 5 TEA-Cl, 5 CsCl, 2 EGTA, 5 QX-314, 2 ATP-Mg and 10 HEPES with a pH adjusted to 7.2 with Tris-base.

Materials

The compounds used in the present study were AlloP, muscimol, tetrodotoxin (TTX), 6-imino-3-(4-methoxyphenyl)-1(6H)-pyridazinebutanoic acid HBr (SR95531), strychnine, kainic acid (KA), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), QX-314, APV, EGTA, bumetanide, picrotoxin, bicuculline, γ-cyclodextrin (γ-CD), finasteride, ZnCl$_2$ and ATP-Mg (from Sigma, St. Louis, MO, USA). All solutions containing drugs were applied using the 'Y–tube system' for rapid solution exchange (Murase et al., 1989).

Results

AlloP acts presynaptically to facilitate spontaneous glutamate release onto hilar neurons

After brief mechanical dissociation of the hippocampal hilar region, large neurons (>20 μm in somatic diameter) that had variously shaped somata, including fusiform, triangular and multipolar forms were observed (see also Lee et al., 2009), which were similar to previously described neurons in the hilar region (Acsády et al., 1998; Johnston and Amaral, 2004). When these neurons were held at a V_H of –60 mV using the whole-cell patch-clamp technique, the spontaneous synaptic currents, including unusually large ones (>100 pA, up to 600 pA), were recorded in the presence of 50 μM APV, a selective NMDA receptor antagonist. These spontaneous currents were completely and reversibly blocked by 20 μM CNQX ($n = 6$), an AMPA/KA receptor blocker, indicating that these spontaneous inward synaptic currents are AMPA/KA receptor-mediated sEPSCs (Figure 1A). The generation of GABAergic spontaneous inhibitory postsynaptic currents was minimized by the use of CsF-based and low Cl$^-$ pipette solution (Lee et al., 2009).

Under these conditions, we first observed the effect of exogenously applied AlloP on glutamatergic sEPSCs in order to elucidate whether the neurosteroid AlloP does affect spontaneous glutamatergic transmission onto hilar neurons. AlloP (100 nM) greatly increased sEPSC frequency, and seemed to increase the frequency of the large sEPSCs in particular (Figure 1A and B). As shown in Figure 1B, AlloP (100 nM) significantly shifted the cumulative distributions of inter-event interval and amplitude of sEPSCs to the left and right, respectively ($P < 0.01$, K-S-test), consistent with increases in the frequency and amplitude of glutamatergic sEPSCs. In 18 neurons in which the AlloP effects on sEPSCs were fully analysed, 100 nM AlloP increased both the frequency (1563 ± 226% of the control, $n = 18$, $P < 0.01$) and amplitude (159 ± 5% of the control, $n = 18$, $P < 0.01$) of glutamatergic sEPSCs (Figure 1B). This increase in sEPSC amplitude was not due to a change in the sensitivity of AMPA/KA receptors by AlloP, as 100 nM AlloP had no effect on the KA (100 μM)-
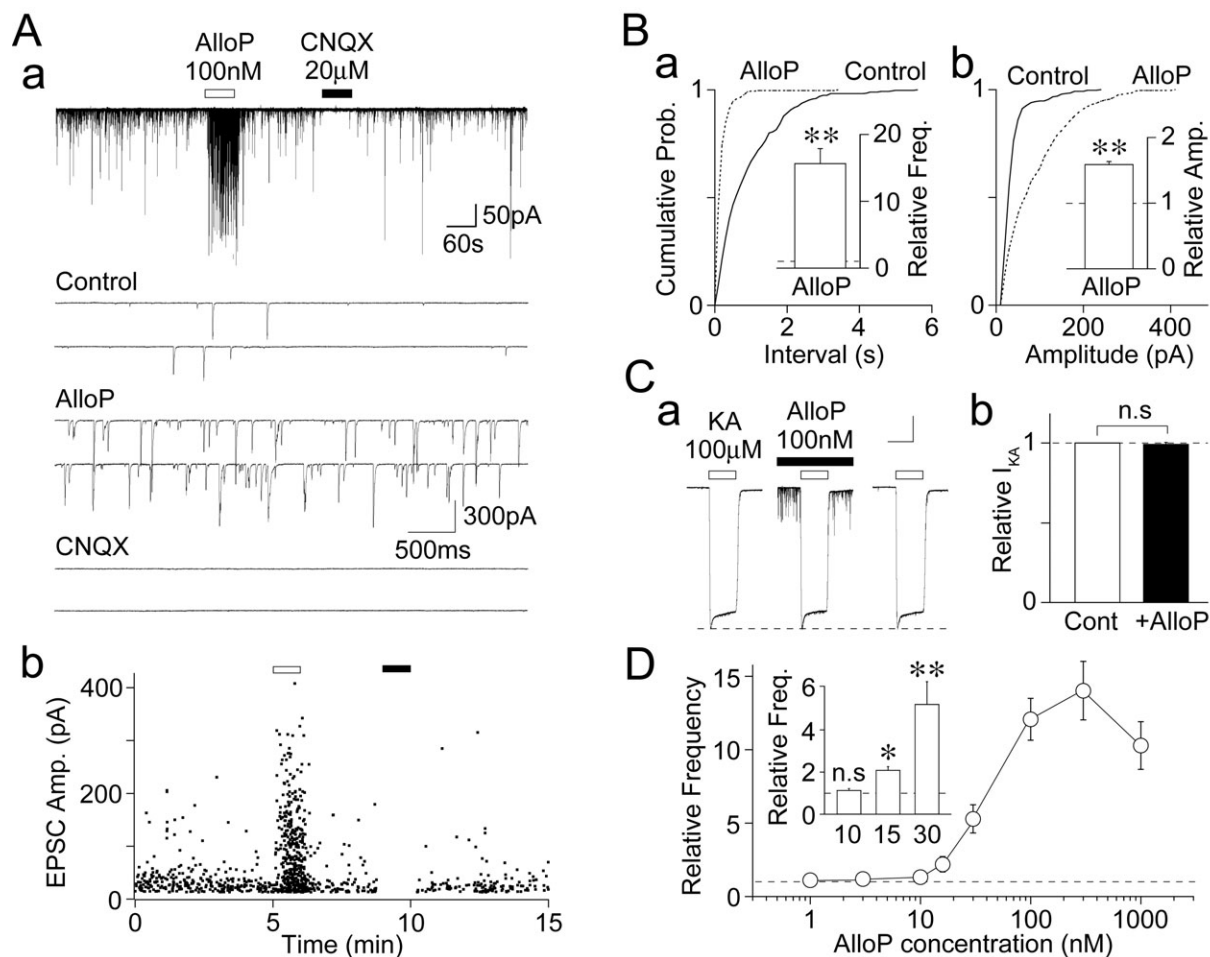
Figure 1
Effect of AlloP on glutamatergic sEPSCs in hilar neurons. (Aa) A typical time course of glutamatergic sEPSC frequency before, during and after the application of 100 nM AlloP or 20 μM CNQX. Insets; typical traces of sEPSCs in the absence (control) and the presence of AlloP or CNQX with an expanded time scale. (Ab) An all points scatter plot of sEPSCs shown in (Aa). 921 events were plotted. (B) Cumulative probability distributions for the inter-event interval (a; $P < 0.01$, K-S test) and current amplitude (b; $P < 0.01$, K-S test) of glutamatergic sEPSCs shown in (A). 401 for the control and 338 events for AlloP were plotted. Insets, 100 nM AlloP-induced changes in sEPSC frequency (left) and amplitude (right). Each column is expressed as the mean ± SEM from 18 experiments. **$P < 0.01$. (Ca) Typical traces of KA (100 μM)-induced currents (I_{KA}) before, during and after the application of 100 nM AlloP (left). (b) Each column and error bar represents the mean ± SEM from five experiments (right). Scale bars; 15 s, 100 pA. n.s, not significant. (D) Concentration–response relationship of sEPSC frequency facilitation against AlloP concentration. Each point and error bar represents the mean ± SEM from 6 to 12 experiments. Inset; AlloP (10, 15, 30 nM)-induced changes in sEPSC frequency. Each column is expressed as the mean ± SEM from 8–16 experiments. n.s, not significant, *$P < 0.05$, *$P < 0.01$.

induced currents (I_{KA}) (Figure 1C). We also examined the concentration–response relationship of AlloP for the sEPSC frequency facilitation (Figure 1D). AlloP at a concentration as low as 15 nM increased sEPSC frequency (204 ± 3% of the control, $n = 16$, $P < 0.05$), but at 10 nM it hardly affected sEPSC frequency (110 ± 6% of the control, $n = 8$, $P = 0.16$, Figure 1D inset). Together, these results suggest that AlloP acts presynaptically to increase spontaneous glutamate release onto mechanically isolated hilar neurons.

AlloP modulation of presynaptic GABA_A receptors
Although AlloP can affect a number of ligand-gated ion channels (Mellon and Griffin, 2002), at nanomolar concentra-

tions, it predominantly affects GABA_A receptors (Majewska et al., 1986; Zhu and Vicini, 1997; Stell et al., 2003; Akk et al., 2005; Hosie et al., 2006). Therefore, we observed the effects of GABA_A receptor antagonists on the AlloP-induced increase in spontaneous glutamate release. SR95531 (10 μM), a competitive GABA_A receptor antagonists, did not change the cumulative distributions of inter-event interval ($P = 0.72$, K-S test) and amplitude ($P = 0.79$, K-S test) of basal sEPSCs (Figure 2A and B). In addition, SR95531 (10 μM) had no effect on the mean frequency (102 ± 5% of the control, $n = 8$, $P = 0.62$) and amplitude (104 ± 7% of the control, $n = 7$, $P = 0.59$) of basal sEPSCs (data not shown). Neither 100 μM SR95531 nor 1 μM strychnine, a glycine receptor blocker, changed the basal frequency of sEPSCs (data not shown). Although the AlloP-induced facilitation of sEPSC frequency was slightly
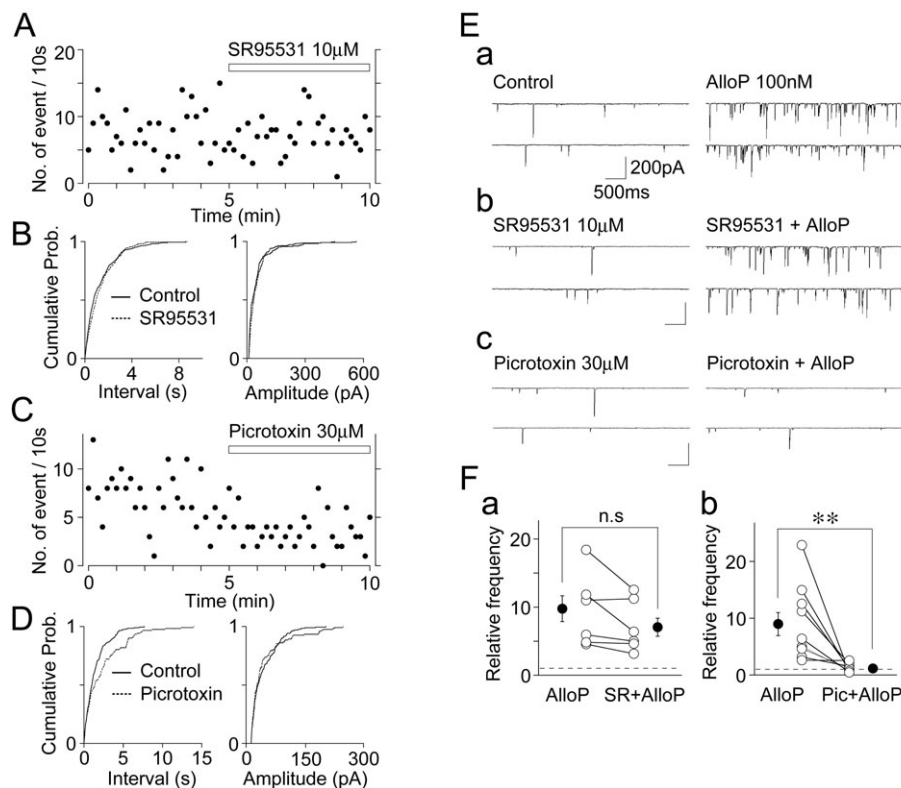
Figure 2

Effects of GABA$_A$ receptor antagonists on the basal frequency of sEPSCs and the AlloP-induced increase in sEPSC frequency. (A) A typical time course of sEPSC frequency before and during the application of 10 μM SR95531. The number of events in every 10 s period was summed and plotted. (B) Cumulative probability distributions for the inter-event interval (left) and amplitude (right) of basal glutamatergic sEPSCs shown in (A). 232 for the control and 223 events for SR95531 were plotted. (C) A typical time course of sEPSC frequency before and during the application of 30 μM picrotoxin. The number of events in every 10 s period was summed and plotted. (D) Cumulative probability distributions for the inter-event interval (left) and amplitude (right) of basal glutamatergic sEPSCs shown in (C). 208 for the control and 122 events for picrotoxin were plotted. (E) Typical traces of sEPSCs recorded before (left) and during (right) the application of 100 nM AlloP in the control external solution (a), in the presence of 10 μM SR95531 (b), and in the presence of 30 μM picrotoxin (c). (F) Changes of the AlloP-induced facilitation of sEPSC frequency in the absence and presence of 10 μM SR95531 (a, $n = 7$) and 30 μM picrotoxin (b, $n = 10$). Open circles and connected lines represent the individual results, whereas closed circles and error bars indicate the mean ± SEM.

reduced in the presence of 10 μM SR95531 (977 ± 189% of the control and 705 ± 133% of the SR95531 condition, $n = 7$, respectively, Figure 2E and Fa), this change was not significant statistically ($P = 0.08$). In contrast, picrotoxin (30 μM), a non-competitive GABA$_A$ receptor blocker, shifted the cumulative distribution of inter-event interval of basal sEPSCs to the right ($P < 0.01$, K-S test), but it did not change the cumulative distribution of the amplitude of basal sEPSCs ($P = 0.22$, K-S test) (Figure 2C and D). In addition, picrotoxin (30 μM) significantly decreased the mean frequency (76 ± 5% of the control, $n = 10$, $P < 0.01$), without affecting the mean amplitude (94 ± 8% of the control, $n = 10$, $P = 0.32$) of basal sEPSCs (data not shown). In the presence of 30 μM picrotoxin, however, the AlloP-induced facilitation of sEPSC frequency (898 ± 203% of the control) was completely prevented ($n = 10$, $P < 0.01$, Figure 2E and Fb). We also investigated whether Zn^{2+} modulated the AlloP-induced increase in spontaneous glutamate release. The application of Zn^{2+} (30 μM) hardly affected the basal sEPSC frequency (97 ± 13% of the control, $n = 6$, $P = 0.56$), as shown in our previous study (Han

et al., 2009). In the presence of 30 μM Zn^{2+}, the AlloP-induced facilitation of sEPSC frequency (1361 ± 320% of the control) was not affected (1109 ± 228% of the values with Zn^{2+} alone, $n = 6$, $P = 0.77$, data not shown).

Because AlloP at low nanomolar concentrations potentiates the GABA$_A$ receptor-mediated currents in postsynaptic neurons (Zhu and Vicini, 1997; Stell et al., 2003; Akk et al., 2005), we investigated whether AlloP potentiates presynaptic GABA$_A$ receptors activated by a GABA$_A$ receptor agonist. In order to test this, we used 10 nM AlloP, in which concentration AlloP had no facilitatory effect on sEPSC frequency as shown in Figure 1D. Muscimol, a GABA$_A$ receptor agonist, increased sEPSC frequency in a concentration-dependent manner as shown in the concentration–response relationship (Figure 3B), where at 100 nM it increased sEPSC frequency (382 ± 49% of the control, $n = 6$, $P < 0.01$, Figure 3A and B). In the presence of 10 nM AlloP, the muscimol (100 nM)-induced increase in sEPSC frequency was significantly increased to 737 ± 126% of the AlloP condition ($n = 6$, $P < 0.05$, Figure 3A and B). In contrast, AlloP reduced the
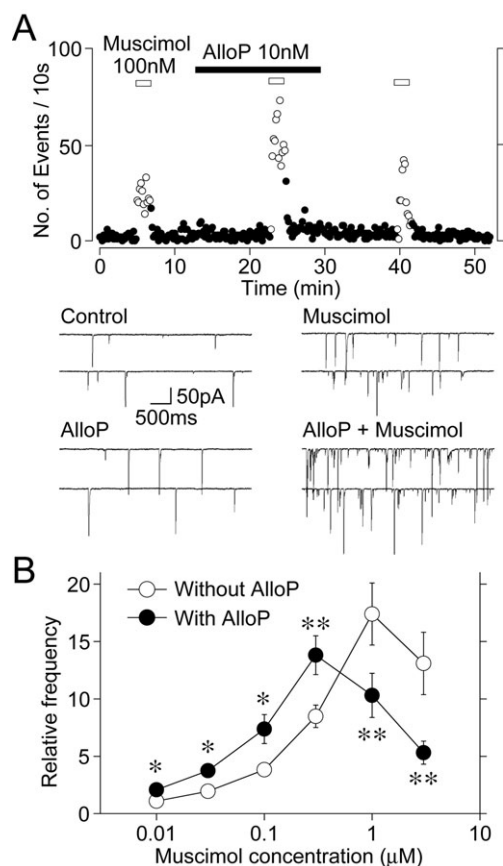
Figure 3

Effect of AlloP on the muscimol-induced increase in sEPSC frequency. (A) A typical time course of sEPSC frequency before, during and after the application of 100 nM muscimol in the absence and presence of 10 nM AlloP. The number of events in every 10 s period was summed and plotted. Inset traces represent typical sEPSPs in numbered regions with an expanded time scale. (B) Changes of the muscimol-induced increase in sEPSC frequency in the absence and presence of 10 nM AlloP. Each point and error bar represents the mean ± SEM from 5–8 experiments. *$P < 0.05$, **$P < 0.01$. Note that AlloP shifted the concentration–response relationship of muscimol to the left.

extent of muscimol (≥ 1 μM)-induced increase in sEPSC frequency (Figure 3B). The data shown in, Figure 3B demonstrates that AlloP shifted the concentration–response relationship of muscimol to the left (Figure 3B), suggesting that AlloP potentiates the action of muscimol on presynaptic GABA$_A$ receptors.

Mechanisms underlying the AlloP-induced facilitation of spontaneous glutamate release

We next examined the mechanisms underlying the AlloP-induced facilitation of sEPSC frequency. The activation of presynaptic GABA$_A$ receptors modulates neurotransmitter release by eliciting a presynaptic depolarization, which activates either voltage-dependent Na$^+$ channels (VDNCs) or voltage-dependent Ca^{2+} channels (VDCCs) (Jang *et al.*, 2001; 2002; 2006). Therefore, we investigated the effects of TTX, a specific VDNC blocker, and Cd^{2+}, a general VDCC blocker, on

the AlloP-induced facilitation of sEPSCs frequency in the presence of 10 μM SR95531. The application of 300 nM TTX significantly decreased the sEPSC frequency ($52 \pm 11\%$ of the control, $n = 8$, $P < 0.01$). In the presence of TTX, however, the action of AlloP on sEPSC frequency was greatly reduced to $189 \pm 56\%$ of the TTX condition ($n = 8$, $P < 0.01$, Figure 4A and B). The application of 100 μM Cd^{2+} also significantly decreased the sEPSC frequency ($43 \pm 12\%$ of the control, $n = 7$, $P < 0.01$). In the presence of Cd^{2+}, the AlloP action on sEPSC frequency was greatly reduced to $94 \pm 14\%$ of the Cd^{2+} condition, $n = 7$, $P < 0.01$, Figure 4A and B). These results suggest that the AlloP-induced activation of presynaptic GABA$_A$ receptors induces a presynaptic depolarization, and subsequently increases Ca^{2+} influx into the presynaptic nerve terminals via VDNCs and VDCCs, thus resulting in the increase of spontaneous glutamate release.

Given that presynaptic GABA$_A$ receptors on glutamatergic nerve terminals induce a presynaptic depolarization that activates VDNCs, the intracellular Cl$^-$ concentration ([Cl$^-$]$_i$) within glutamatergic nerve terminals should be maintained at a higher level than that predicted for passive Cl$^-$ distribution. This would be mediated by inwardly directed Cl$^-$ co-transporters such as Na-K-Cl co-transporter type 1 (NKCC1), as reported previously (Jang *et al.*, 2001; 2002; Lee *et al.*, 2009). To confirm that AlloP indeed induces a presynaptic depolarization via a similar mechanism, we tested the effect of bumetanide, a selective NKCC1 blocker (Haas, 1989), on the AlloP-induced facilitation of sEPSC frequency (Figure 4C and D). The extent of AlloP-induced facilitation of sEPSC frequency ($1805 \pm 462\%$ of the control, $n = 5$, $P < 0.05$) was gradually attenuated in the prolonged presence of 10 μM bumetanide (3rd application: $168 \pm 62\%$, $n = 5$, $P = 0.43$). However, the effect of AlloP slowly recovered after the washout of bumetanide (30 min later: $1343 \pm 126\%$ of the control, $n = 5$, $P < 0.01$, Figure 4C and D).

Presynaptic GABA$_A$ receptors are tonically activated by endogenous neurosteroids

We next examined whether presynaptic GABA$_A$ receptors are tonically activated by endogenous 3α-hydroxy, 5α-reduced pregnane steroids, because both 5α-reductase and 3α-HSD are exclusively expressed on hippocampal glutamatergic neurons (Agís-Balboa *et al.*, 2006). In order to evaluate this hypothesis, we investigated the effects of γ-CD (a membrane-impermeable and efficient sequestering agent (which binds several types of naturally occurring neurosteroids including AlloP and THDOC; Shu *et al.*, 2007), on the basal frequency of sEPSCs in the presence of 10 μM SR95531. If endogenous 3α-hydroxy, 5α-reduced pregnane steroids act on presynaptic GABA$_A$ receptors to facilitate the frequency of basal sEPSCs, exogenously applied γ-CD would be expected to decrease the frequency of basal sEPSCs. As expected, the application of 500 μM γ-CD significantly shifted the cumulative distribution of inter-event interval sEPSCs to the right ($P < 0.01$, K-S test) and decreased the mean frequency of basal sEPSCs to $64 \pm 14\%$ of the control ($n = 7$, $P < 0.01$) (Figure 5Ba). However, γ-CD did not change the cumulative distribution ($P = 0.21$, K-S test) and the mean amplitude ($96 \pm 7\%$ of the control, $n = 7$, $P = 0.72$) of sEPSCs (Figure 5Bb). In the presence of 500 μM γ-CD the AlloP (100 nM)-induced facilitation
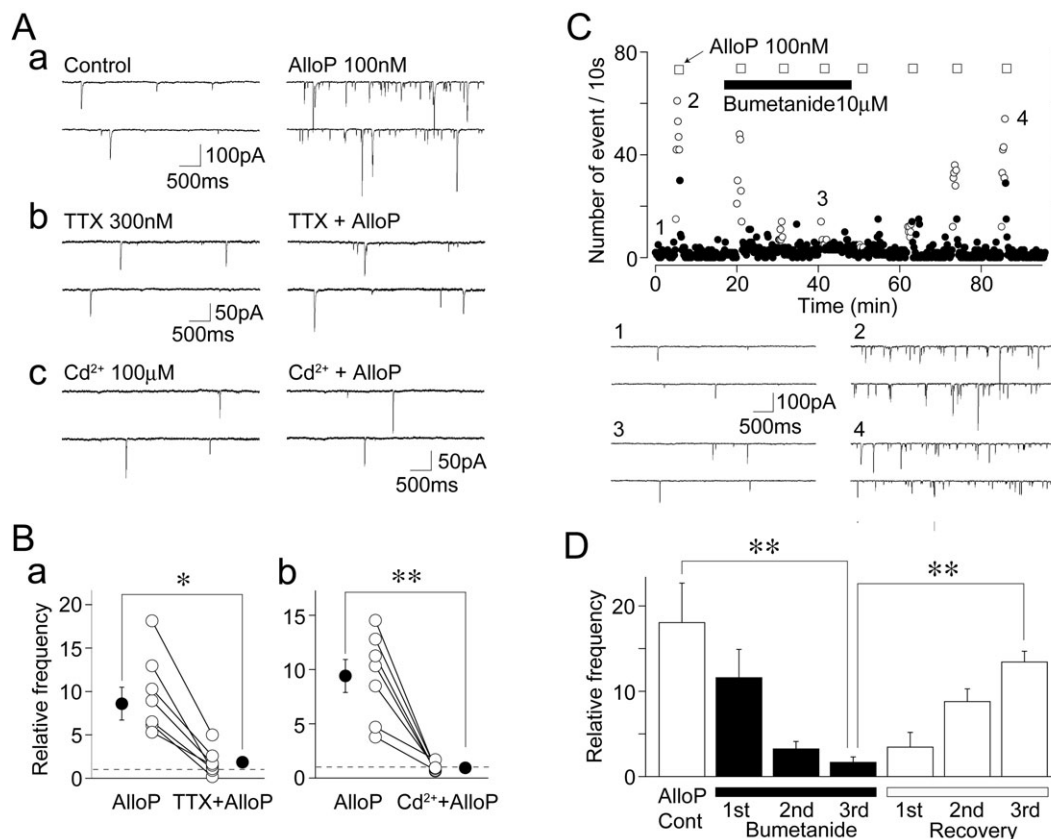
Figure 4

AlloP depolarizes glutamatergic nerve terminals. (A) Typical traces of sEPSCs recorded before (left) and during (right) the application of 100 nM AlloP in the control external solution (a), in the presence of 300 nM TTX (b) and in the presence of 100 μM Cd^{2+} (c). (B) The changes in AlloP-induced increase in sEPSC frequency in the presence of TTX (a) or Cd^{2+} (b). Each column and error represents was the mean and SEM from 8 for TTX and 7 for Cd^{2+} experiments. Open circles and connected lines represent the individual results, whereas closed circles and error bars indicate the mean ± SEM. *$P < 0.05$, **$P < 0.01$. (C) A typical time course of sEPSC frequency before, during and after the application of 100 nM AlloP in the absence and presence of 10 μM bumetanide. The number of events in every 10 s period was summed and plotted. Inset traces represent typical sEPSPs in the numbered regions with an expanded time scale. (D) Changes of the AlloP-induced increase in sEPSC frequency in the absence and presence of bumetanide. Each column and error bar represents the mean ± SEM from five experiments. **$P < 0.01$.

of sEPSC frequency (735 ± 144% of the control, $n = 7$) was completely attenuated to 140 ± 23% of the γ-CD condition ($n = 7$, $P < 0.01$, Figure 5C), ascertaining that γ-CD efficiently sequesters AlloP.

To confirm whether presynaptic GABA$_A$ receptors are involved in the decrease in sEPSC frequency induced by γ-CD, we examined the effect of picrotoxin on γ-CD-induced decrease in basal sEPSC frequency in the presence of 10 μM SR95531, because picrotoxin itself decreased the basal frequency of sEPSCs as shown in Figure 2. In presence of 30 μM picrotoxin, γ-CD (500 μM) failed to decrease basal sEPSC frequency (94 ± 10% of the picrotoxin treatment, $n = 10$, $P = 0.23$, Figure 6A and D). We also examined the effect of TTX on γ-CD-induced decrease in basal sEPSC frequency, because TTX completely blocked the AlloP-induced increase in sEPSC frequency as described above. In the presence of 300 nM TTX, γ-CD (500 μM) again failed to decrease basal sEPSC frequency (100 ± 5% of the TTX condition, $n = 10$, $P = 0.46$, Figure 6B and D). In order to determine whether γ-CD decreased the basal frequency of sEPSCs after

the synthesis of endogenous 3α-hydroxy, 5α-reduced pregnane steroids was blocked, we examined the effect of finasteride, a specific 5α-reductase inhibitor, on the basal frequency of sEPSCs. However, the application of 10 μM finasteride for 10 min did not change the basal sEPSC frequency (97 ± 12% of the control, $n = 5$, $P = 0.56$, data not shown). This observation might be due to a slow (>1 h) neosynthesis of endogenous 5α-reduced pregnane steroids, as shown in a previous study (Keller *et al.*, 2004). Therefore, we incubated hippocampal slices with 10 μM finasteride immediately after making the slices, and examined the effect of γ-CD on the basal frequency of sEPSCs. In the continued presence of 10 μM finasteride, γ-CD (500 μM) failed to decrease the basal frequency of sEPSCs (100 ± 5% of the finasteride condition, $n = 7$, $P = 0.61$, Figure 6C and D). Together, these results suggest that endogenous neurosteroids such as AlloP and THDOC, which can be trapped by extracellularly applied γ-CD, might contribute to the tonic activation of presynaptic GABA$_A$ receptors expressed on glutamatergic nerve terminals.
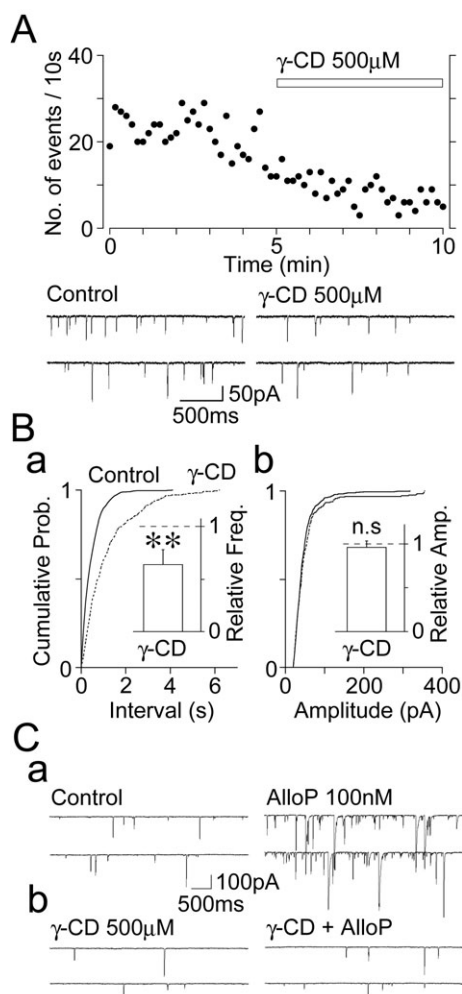
Figure 5

Effects of γ-CD on the basal frequency of sEPSCs. (A) A typical time course of sEPSC frequency before and during the application of 500 μM γ-CD in the control condition. The number of events in every 10 s period was summed and plotted. Insets; typical traces of sEPSCs in the absence (control) and the presence of γ-CD with an expanded time scale. (B) Cumulative probability distribution for the inter-event interval (a) and amplitude (b) of glutamatergic sEPSCs shown in (A). 664 for the control and 261 events for γ-CD were plotted. Insets, the γ-CD-induced changes in the basal frequency (a) and amplitude (b) of sEPSCs. Columns are the mean ± SEM from seven experiments. **$P < 0.01$. (C) Typical traces of sEPSCs recorded before (left) and during (right) the application of 100 nM AlloP in the control external solution (a) and in the presence of 500 μM γ-CD (b).

Our previous studies have shown that presynaptic GABA$_A$ receptors expressed on hippocampal glutamatergic nerve terminals are highly sensitive to muscimol (Jang *et al.*, 2006; Han *et al.*, 2009). Based on the present results showing that AlloP potentiates the muscimol-induced increase in spontaneous glutamate release (Figure 3) and that endogenous 3α-hydroxy, 5α-reduced pregnane steroids might modulate presynaptic GABA$_A$ receptors (Figures 5 and 6), it is possible that the higher sensitivity of presynaptic GABA$_A$ receptors to muscimol might be due to the intrinsic modulation of GABA$_A$ receptors by endogenous neurosteroids. To test this hypoth-
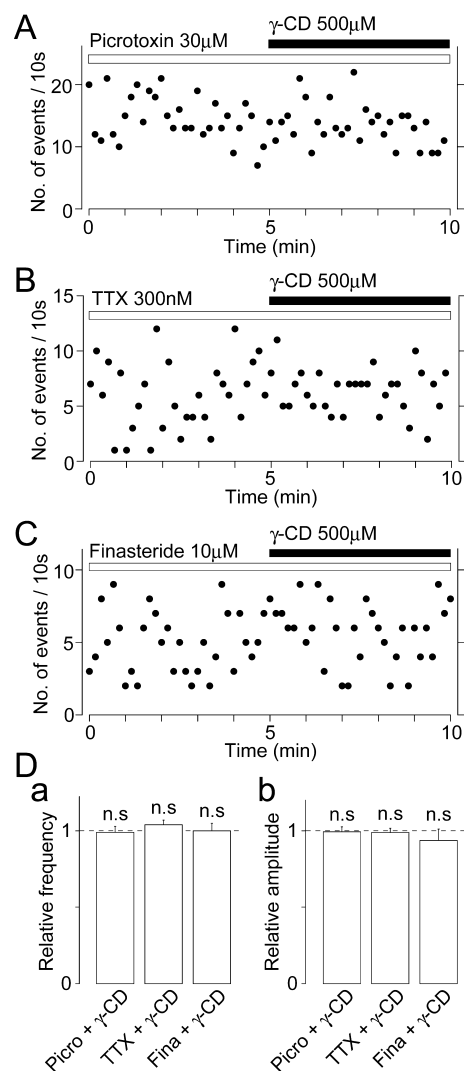
Figure 6

Effects of picrotoxin, TTX and finasteride on the γ-CD-induced decrease in basal sEPSC frequency. (A) A typical time course of sEPSC frequency before and during the application of 500 μM γ-CD in the presence of 30 μM picrotoxin. The number of events in every 10 s period was summed and plotted. (B) A typical time course of sEPSC frequency before and during the application of 500 μM γ-CD in the presence of 300 nM TTX. The number of events in every 10 s period was summed and plotted. (C) A typical time course of sEPSC frequency before and during the application of 500 μM γ-CD in the presence of 10 μM finasteride. The number of events in every 10 s period was summed and plotted. Note that finasteride (10 μM) was added in the incubation solution immediately after making hippocampal slices. (D) Changes of the basal frequency (a) and amplitude (b) of sEPSCs by γ-CD in the absence and presence of 30 μM picrotoxin (Picro; $n = 10$), 300 nM TTX ($n = 10$) and 10 μM finasteride (Fina; $n = 7$).

esis, we examined the effect of γ-CD on the muscimol-induced increase in spontaneous glutamate release. The muscimol (100 nM)-induced increase in sEPSC frequency (353 ± 34% of the control, $n = 8$) was significantly reduced in the presence of 500 μM γ-CD (230 ± 29% of the γ-CD condi-
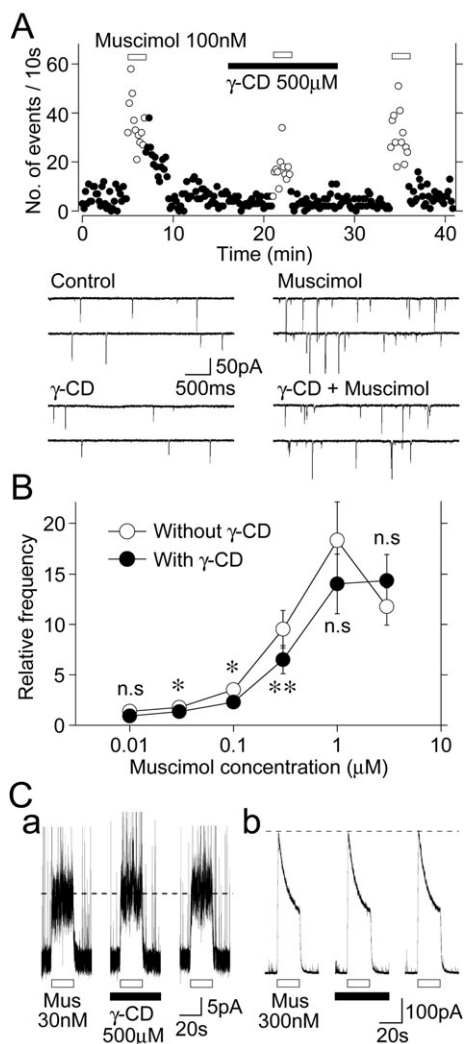
Figure 7

Effects γ-CD on the muscimol-induced increase in sEPSC frequency. (A) A typical time course of sEPSC frequency before, during and after the application of 100 nM muscimol in the absence and presence of 500 μM γ-CD. The number of events in every 10 s period was summed and plotted. Inset traces represent typical sEPSPs in numbered regions with an expanded time scale. (B) Changes of the muscimol-induced increase in sEPSC frequency in the absence and presence of 500 μM γ-CD. Each point and error bar represents the mean ± SEM from 5–8 experiments. *$P < 0.05$, **$P < 0.01$. Note that γ-CD shifted the concentration–response relationship of muscimol to the right. (C) Representative traces of I_{Mus} induced by 30 nM (a) or 300 nM muscimol (Mus; b) in the absence and presence of 500 μM γ-CD. Muscimol was applied at 4 min intervals and γ-CD was preincubated 1 min before the muscimol application. Note that γ-CD had no effect on the I_{Mus}.

tion, $n = 8$, $P < 0.05$, Figure 7A and B). However, γ-CD had no inhibitory effect on the muscimol (≥ 1 μM)-induced increase in sEPSC frequency (Figure 7B). These data indicate that γ-CD shifted the concentration–response relationship of muscimol to the right (Figure 7B). On the other hand, it is possible that γ-CD directly inhibits GABA$_A$ receptors and thus it attenuates the facilitatory action of muscimol on sEPSC frequency.

Therefore, we examined the direct effect of γ-CD on GABA$_A$ receptors in acutely isolated DG granule cells, which are parent neurons of mossy fibres. However, γ-CD (500 μM) had no inhibitory effect on the I_{Mus} induced by 30 nM and 300 nM muscimol (Figure 7C), suggesting that γ-CD had no inhibitory effect on GABA$_A$ receptors themselves.

Effects of AlloP and γ-CD on action potential-dependent glutamate release

To elucidate the physiological significance of the present findings, we examined the effect of AlloP on action potential-dependent glutamate release in the slice preparation. Glutamatergic eEPSCs were recorded in the presence of 10 μM SR95531 and 50 μM APV. In six of nine neurons tested, bath applied AlloP (100 nM) slightly but significantly increased the amplitude of eEPSCs (120 ± 4% of the control, $n = 6$, $P < 0.05$) and decreased the paired-pulse ratio (PPR) from 1.65 ± 0.08 to 1.52 ± 0.07 (92 ± 1% of the control, $n = 6$, $P < 0.01$, Figure 8A and C), suggesting that AlloP acts presynaptically to increase the probability of glutamate release from mossy fibre terminals. In the remaining three neurons, AlloP had no facilitatory effect on glutamatergic eEPSCs. In a subset of experiments, we also observed the effect of γ-CD on action potential-dependent glutamate release. In 7 of 11 neurons tested, bath applied γ-CD (500 μM) significantly decreased the amplitude of eEPSCs (82 ± 4% of the control, $n = 7$, $P < 0.05$) and increased the PPR from 1.60 ± 0.04 to 1.91 ± 0.07 (117 ± 5% of the control, $n = 7$, $P < 0.05$, Figure 8B and C). In the remaining four neurons, γ-CD had no inhibitory effect on glutamatergic eEPSCs. The results suggest that endogenous neurosteroids, which can be sequestered by γ-CD, act on presynaptic GABA$_A$ receptors to decrease the probability of glutamate release from mossy fibre terminals.

Discussion

In the present study, we found that AlloP acts presynaptically to increase sEPSC frequency on acutely isolated hilar neurons. Although AlloP also increased the mean amplitude of sEPSCs, the AlloP-induced increase in sEPSC amplitude might be due to an increase in the frequency of large sEPSCs (see also Jang *et al.*, 2006). The facilitatory action of AlloP should result from the direct activation of presynaptic GABA$_A$ receptors rather than the interaction of agonist binding sites, because the AlloP action was significantly blocked by noncompetitive rather than competitive GABA$_A$ receptor antagonists. Although a few studies have shown that the neurosteroid-mediated Cl$^-$ currents are sensitive to SR95531, such inhibitory effects have been tested for the Cl$^-$ currents induced by higher (≥ 300 nM) concentrations of neurosteroids (Ueno *et al.*, 1997; Shu *et al.*, 2004). Similarly, we have recently shown that while SR95531 significantly reduced the Cl$^-$ currents induced by 1 μM AlloP, it had no inhibitory effect on the Cl$^-$ currents induced by ≤ 100 nM AlloP (Park *et al.*, 2011). In addition, the AlloP-induced increase in sEPSC frequency was hardly affected by 30 μM Zn^{2+}, as presynaptic GABA$_A$ receptors expressed on putative mossy fibre terminals are highly insensitive to Zn^{2+} (Han *et al.*, 2009). These phar-
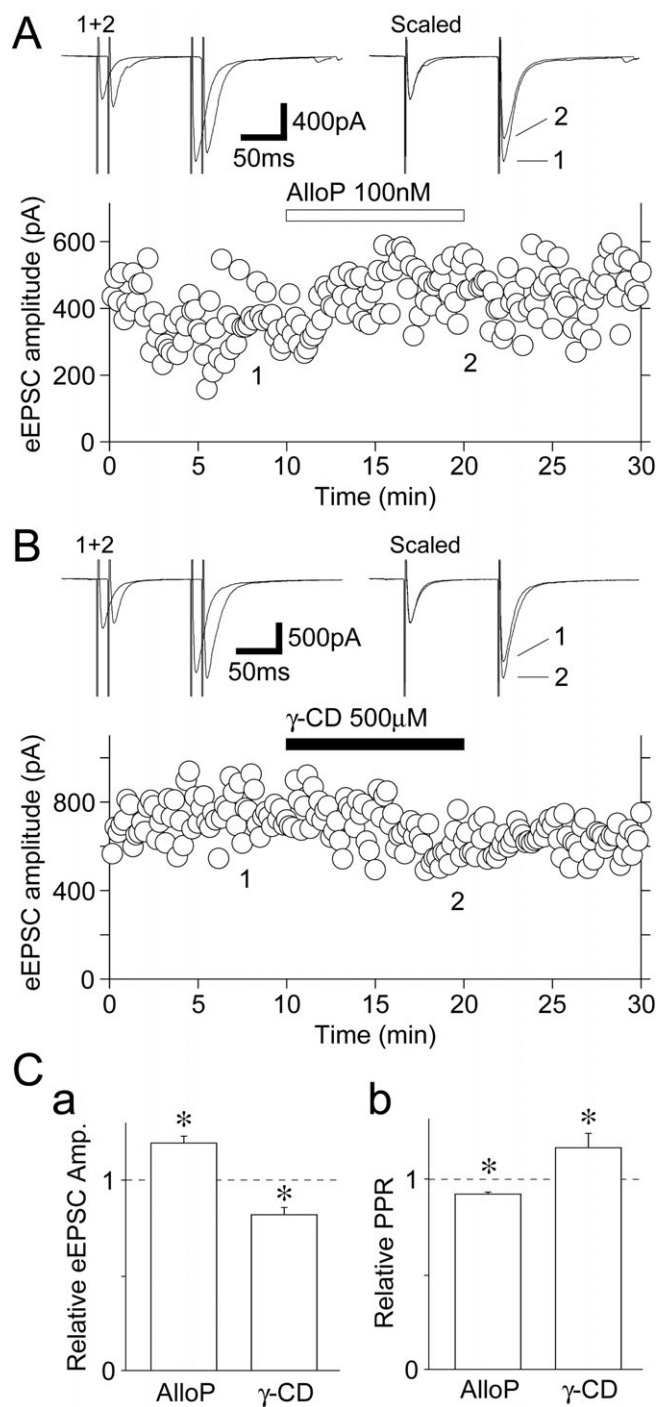
Figure 8

Effects of AlloP and γ-CD on eEPSCs in the slice preparation. (A) A typical time course of eEPSC amplitude before, during and after application of 100 nM AlloP. Inset traces represent typical eEPSCs in numbered regions with an expanded time scale. (B) A typical time course of eEPSC amplitude before, during and after application of 500 μM γ-CD. Inset traces represent typical eEPSCs in numbered regions with an expanded time scale. (C) AlloP- and γ-CD-induced changes in the amplitude of eEPSCs (a) and the PPR (b). Each column and error bar represents the mean ± SEM from 6–7 experiments. *$P < 0.05$.

macological data strongly suggest that AlloP at nanomolar concentrations directly activates presynaptic GABA$_A$ receptors to facilitate spontaneous glutamate release onto hilar neurons. Although the AlloP concentration for direct GABA$_A$ receptor activation is in a range of 300 nM to 10 μM, which is substantially higher than that for the modulatory action (Belelli *et al.*, 2002; Shu *et al.*, 2004; Hosie *et al.*, 2006), our recent study has shown that AlloP at ≥30 nM concentrations can elicit the Cl$^-$ currents in hippocampal neurons (Park *et al.*, 2011). On the other hand, AlloP also increased the muscimol-induced facilitation of sEPSC frequency and shifted the concentration–response relationship to the left. Taken together, it is likely that AlloP, even at low nanomolar concentrations, has dual effects (direct activation and potentiation) on presynaptic GABA$_A$ receptors.

Given that AlloP directly activates presynaptic GABA$_A$ receptors as described above, AlloP should depolarize the terminal membrane as the activation of presynaptic GABA$_A$ receptors, which elicits the Cl$^-$ conductance, is also known to depolarize mossy fibres (Ruiz *et al.*, 2003; Jang *et al.*, 2006; Nakamura *et al.*, 2007). In the present study, the AlloP-induced increase in sEPSC frequency was completely blocked by either TTX or Cd^{2+}, suggesting that AlloP directly depolarizes the terminal membrane and contributes to the activation of VDNCs and VDCCs. As a result, the Ca^{2+} influx through presynaptic VDCCs would be responsible for the AlloP-induced increase in spontaneous glutamate release. If AlloP depolarizes the terminal membrane via the direct activation of presynaptic GABA$_A$ receptors, the Cl$^-$ equilibrium potential must be positive with respect to the resting membrane potential of presynaptic terminals, that is, glutamatergic nerve terminals innervating hilar neurons should have a higher intraterminal [Cl$^-$] than predicted for passive distribution. This effect could be accomplished by inwardly directed Cl$^-$ co-transporters, such as NKCC1 (Plotkin *et al.*, 1997; Clayton *et al.*, 1998; Jang *et al.*, 2001; 2002). In the present study, the AlloP-induced increase in sEPSC frequency was gradually reduced in the presence of bumetanide, a specific NKCC1 blocker (Haas, 1989), which has no inhibitory effect on GABA$_A$ receptors (Jang *et al.*, 2001). These results suggest that bumetanide-sensitive NKCC1 contributes to the AlloP-induced presynaptic depolarization of glutamatergic nerve terminals projecting to hilar neurons.

One important aim of this study was to elucidate the possible roles of endogenous neurosteroids in presynaptic GABA$_A$ receptor function. In the present study, we found that γ-CD decreased the basal frequency of sEPSCs, and that non-competitive but not competitive GABA$_A$ receptor antagonists reduced the basal frequency of sEPSCs. In addition, as the γ-CD did not affect the basal frequency of sEPSCs in the presence of TTX or after blocking the synthesis of 5α-reduced neurosteroids, both endogenous neurosteroids and GABA$_A$ receptors seem to be closely related to basal spontaneous glutamate release. The depletion of membrane cholesterol rather than neurosteroids by γ-CD changes the probability of spontaneous glutamate release. However, γ-CD is less effective than β-CD in sequestering cholesterol from membranes (Ohtani *et al.*, 1989). A previous study has also shown that the depletion of cholesterol by methyl-β-CD increases, rather than decreases, Ca^{2+}-dependent asynchronous transmitter release (Zamir and Charlton, 2006). These results suggest that

endogenous 3α-hydroxy, 5α-reduced pregnane steroids act on presynaptic GABA$_A$ receptors to change the excitability of glutamatergic nerve terminals and regulate the probability of basal spontaneous glutamate release. On the other hand, the origin of endogenous AlloP in mechanically isolated hippocampal neurons is unknown. Given that both 3α-hydroxy, 5α-reduced pregnane steroids and 5α-reductase are strongly expressed in glutamatergic principal neurons including DG granule cells but not hilar neurons (Agís-Balboa *et al.*, 2006; Saalmann *et al.*, 2007), endogenous neurosteroids might be synthesized in glutamatergic nerve terminals rather than postsynaptic hilar neurons. Although the actual concentration of endogenous AlloP around glutamatergic nerve terminals remains to be elucidated, other neuroactive steroids having a positive effect on GABA$_A$ receptors, such as THDOC or 3α-hydroxy, 5β-reduced pregnane steroids, might synergistically act on presynaptic GABA$_A$ receptors to affect basal glutamatergic transmission.

The higher sensitivity of presynaptic GABA$_A$ receptors to muscimol might be due to the subunit composition of GABA$_A$ receptors, as GABA$_A$ receptors containing α5 or δ subunits are known to be more sensitive to GABA (Mtchedlishvili and Kapur, 2006). Previous reports have shown that δ subunit-containing GABA$_A$ receptors, especially α4β2δ subunit combinations, have a high sensitivity to GABA (Sundstrom-Poromaa *et al.*, 2002) and might contribute to the tonic inhibition of DG granule cells (Wei *et al.*, 2003). In addition, δ subunit-containing GABA$_A$ receptors are more sensitive to 3α-hydroxy, 5α-reduced pregnane steroids (Stell *et al.*, 2003). However, our previous study has suggested that presynaptic GABA$_A$ receptors expressed on hippocampal glutamatergic nerve terminals presumably contain γ subunits, according to the diazepam and Zn^{2+} sensitivities (Han *et al.*, 2009). For example, γ subunit-containing GABA$_A$ receptors are much less sensitive to Zn^{2+} (α1β1; IC$_{50}$: 1.2 μM, α1β1γ2; IC$_{50}$: >600 μM, α1β1δ; IC$_{50}$: 16.3 μM) (Draguhn *et al.*, 1990; Krishek *et al.*, 1998; Brown *et al.*, 2002; Mtchedlishvili and Kapur, 2006). In addition, DG granule cells express multiple types of GABA$_A$ receptors, such as α1βγ2, α4βγ2 and α4βδ GABA$_A$ receptors (Wei *et al.*, 2003; Lindquist and Birnir, 2006). In contrast, α5 subunit-containing GABA$_A$ receptors would contribute to the higher sensitivity of presynaptic GABA$_A$ receptors as DG granule cells are likely to express α5 subunits (Glykys *et al.*, 2008; but see also Sperk *et al.*, 1997). In the present study, we also found that γ-CD decreased the extent of muscimol-induced increase in sEPSC frequency and shifted the concentration–response relationship of muscimol to the right, suggesting that γ-CD reduces the sensitivity of presynaptic GABA$_A$ receptors to muscimol. The depletion of membrane cholesterol by γ-CD can also change the sensitivity of presynaptic GABA$_A$ receptors to muscimol, as cholesterol depletion lowers the EC$_{50}$ value of GABA (Sooksawate and Simmonds, 2001a). However, the depletion of membrane cholesterol by methyl-β-CD increases the potentiation of GABA by pregnanolone (Sooksawate and Simmonds, 2001b). Furthermore, we found that γ-CD had no effect on GABA$_A$ receptor-mediated Cl$^-$ currents in DG granule cells, suggesting that γ-CD has no direct inhibitory effect on GABA$_A$ receptors themselves. Given that endogenous neurosteroids act on presynaptic GABA$_A$ receptors as described above, it is highly feasible that endogenous 3α-hydroxy, 5α-reduced pregnane

steroids contribute, at least in part, to the higher sensitivity of presynaptic GABA$_A$ receptors to agonists.

The hippocampus is closely related to a variety of pathophysiological conditions including stress, epilepsy, and learning and memory (Bliss and Collingridge, 1993; Coulter, 2000). In the hippocampus, mossy fibres arising from DG granule cells innervate hilar neurons, excitatory mossy cells and inhibitory basket cells, before entering the CA3 region of the hippocampus (Acsády *et al.*, 1998; Johnston and Amaral, 2004), suggesting that a significant portion of sEPSCs recorded from acutely isolated hilar neurons may originate from mossy fibres (see also Lee *et al.*, 2009). Mossy fibre-derived spontaneous excitatory postsynaptic potentials (sEPSPs) are sufficiently large to trigger action potentials in postsynaptic neurons (Henze *et al.*, 2002; Jang *et al.*, 2006), and hilar neurons are known to receive large sEPSPs that can trigger bursts of action potentials (Scharfman and Schwartzkroin, 1988). In this regard, our present results suggest that AlloP through the direct activation and/or potentiation of presynaptic GABA$_A$ receptors might have an excitatory effect on postsynaptic hilar neurons because AlloP greatly increased sEPSC frequency, especially the frequency of large sEPSCs. However, considering that mossy fibres project to more inhibitory interneurons than do excitatory neurons in the DG as well as CA3 regions (Seress *et al.*, 2001), and that neuronal death in the hilar region induces the hyperexcitability of hippocampal neural networks (McNamara, 1994), the functional roles of endogenous neurosteroids in the modulation of glutamatergic transmission is likely to be complex in the context of the intrinsic hippocampal circuitry.

In conclusion, our present results suggest that presynaptic GABA$_A$ receptors and their activation and/or potentiation by endogenous neurosteroids might affect the excitability of the DG-hilus-CA3 network, and thus contribute, at least in part, to some pathological conditions, such as catamenial epilepsy and premenstrual dysphoric disorder.

Acknowledgements

Conflicts of interest

None.

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