Neurosteroid modulation of benzodiazepine-sensitive GABA<sub>A</sub> tonic inhibition in supraoptic magnocellular neurons

Ji Yoon Jo,1 Ji Ae Jeong,1 Sudip Pandit,1 Javier E. Stern,2 Seul Ki Lee,4 Pan Dong Ryu,4 So Yeong Lee,4 Seong Kyu Han,5 Chung-Hyun Cho,1 Hyun Woo Kim,1,2 Byeong Hwa Jeon,1 and Jin Bong Park1,2

1Department of Physiology and 2Brain Research Institute, School of Medicine, Chungnam National University, Daejeon, Korea; 3Department of Physiology, Medical College of Georgia, Augusta, Georgia; 4Department of Pharmacology, College of Veterinary Medicine, Seoul National University, Seoul, Korea; and 5Department of Oral Physiology, School of Dentistry and Institute of Oral Bioscience, Chonbuk National University, Jeonju, Korea

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Neurosteroid modulation of benzodiazepine-sensitive GABA<sub>A</sub> tonic inhibition in supraoptic magnocellular neurons. Am J Physiol Regul Integr Comp Physiol 300: R1578–R1587, 2011. First published March 30, 2011; doi:10.1152/ajpregu.00627.2010.—Interactions between neurosteroids and GABA receptors have attracted particular attention in the supraoptic nucleus (SON). Although GABA<sub>A</sub> receptors (GABA<sub>A</sub>R) mediate a sustained tonic inhibitory current (I<sub>tonic</sub>), as well as conventional phasic inhibitory postsynaptic currents (IPSCs, I<sub>phasic</sub>), the molecular configuration of GABA<sub>A</sub> receptors mediating I<sub>tonic</sub> and I<sub>phasic</sub> in the SON, whether the steroid modulation on I<sub>tonic</sub> is present in SON magnocellular neurosecretory cells (MNCs) is unknown. Here, we addressed this question and gained insights into the potential molecular configuration of GABA<sub>A</sub> receptors underlying persistent tonic inhibitory currents (I<sub>tonic</sub>), as well as conventional phasic inhibitory postsynaptic currents (IPSCs, I<sub>phasic</sub>) in the SON, whether the steroid modulation on I<sub>tonic</sub> is present in SON magnocellular neurosecretory cells (MNCs) is unknown. Here, we addressed this question and gained insights into the potential molecular configuration of GABA<sub>A</sub> receptors mediating I<sub>tonic</sub> and conferring its neurosteroids sensitivity in SON MNCs. 4,5,6,7-tetrahydroisoxazolo[5,4-c]-pyridin-3-ol (THIP) (1 μM), a relatively selective extrasynaptic GABA<sub>A</sub>R agonist, facilitated I<sub>tonic</sub> without affecting the main characteristics of IPSCs, while DS-2, a relatively selective modulator of GABA<sub>A</sub>R α<sub>5</sub>-subunits, caused minimal changes in I<sub>tonic</sub> of SON MNCs. L-655,708, a relatively selective GABA<sub>A</sub>R α<sub>5</sub>-subunit inverse agonist, blocked ~35% of the total I<sub>tonic</sub> both under basal and elevated ambient GABA concentration (3 μM). Facilitation of I<sub>tonic</sub> by benzodiazepines further supported the role of GABA<sub>A</sub>R γ<sub>2</sub>-subunit in I<sub>tonic</sub> of SON MNCs. Quantitative RT-PCR analysis showed much lesser expression of GABA<sub>A</sub>R γ<sub>2</sub>-subunit than the α<sub>5</sub> or γ<sub>2</sub>-subunit in the SON. Allopregnanolone and 3α,5α-tetrahydrodeoxy corticosterone increased both I<sub>tonic</sub> and I<sub>phasic</sub> in SON MNCs, respectively, although more than 90% of the current increase was mediated by I<sub>tonic</sub> during the neurosteroid facilitation. Finally, L-655,708 attenuated the neurosteroid facilitation of I<sub>tonic</sub> but not of I<sub>phasic</sub>. Altogether, our results suggest that I<sub>tonic</sub>, mediated mainly by benzodiazepine-sensitive GABA<sub>A</sub>Rs containing α<sub>5</sub>-, β-, and γ<sub>2</sub>-, and to a lesser extent, δ-subunits, is a potential target of neurosteroid modulation in SON neurons.

GABA<sub>A</sub> receptors; oxytocin; vasopressin; extrasynaptic; neurosteroid action of SON neurons acts on the neurons to reduce the efficacy of GABA actions, and this effect is blocked by the neurosteroid allopregnanolone (3α,5α-THP). At term pregnancy, the fall in progesterone precipitates enhanced excitability of oxytocin neurons through this effective GABA disinhibition (7, 9).

GABA<sub>A</sub>R underlie persistent tonic inhibitory currents (I<sub>tonic</sub>), as well as conventional inhibitory postsynaptic currents (IPSCs, I<sub>phasic</sub>) in the central nervous system (15, 25, 44). GABA<sub>A</sub>R mediating I<sub>basic</sub> are activated by brief exposure to a high concentration of the neurotransmitter, while the receptors mediating I<sub>tonic</sub> are activated by low ambient concentration of the transmitter in the extracellular space. I<sub>tonic</sub>, originally known in cerebellar (CGCs) and dentate gyrus (DGCCs) granule cells, is mediated by GABA<sub>A</sub>R containing δ-subunit associated with the α<sub>6</sub>-subunit (22, 28) and the α<sub>4</sub>-subunit (48), respectively. I<sub>tonic</sub>, mediated by δ-subunit-containing GABA<sub>A</sub>R<sub>M</sub>, appears more sensitive to neurosteroids than its synaptic counterpart, I<sub>phasic</sub>, which is mediated by γ<sub>2</sub>-subunit-containing receptors. For example, I<sub>tonic</sub> is selectively enhanced by a low concentration of 3α,5α-THDOC that has no effect upon the kinetics of I<sub>phasic</sub> in DGCCs and CGCs (46). Facilitation of I<sub>phasic</sub> has been considered the primary mechanism whereby neurosteroids influence neuronal excitability in SON MNCs (8, 14, 23). However, GABA<sub>A</sub>R of possibly different molecular configuration mediate I<sub>tonic</sub>, as well as I<sub>phasic</sub> in SON MNCs (32). Despite the wealth of information available on I<sub>phasic</sub> no information is available so far on the neurosteroids modulation of I<sub>tonic</sub> in SON MNCs. Even whether the steroid modulation on I<sub>tonic</sub> is present in the neurons is unknown. In this study, we obtained information on the molecular configuration of GABA<sub>A</sub> receptors underlying the steroid modulation of I<sub>tonic</sub> and showed the major role of I<sub>tonic</sub> in pregnant steroids potentiation of GABA<sub>A</sub> inhibition in SON MNCs.

MATERIALS AND METHODS

Experimental animals. Male Sprague-Dawley rats (5–6 wk, 130–180 g) were purchased and housed in a 12:12-h light-dark schedule and allowed free access to food and water. All animal experimentation was conducted under the license (2009–1-21) issued by the Animal Ethics Committee of Chungnam National University and was in compliance with the policy of Chungnam National University regarding the use and care of animals.

Electrophysiological recordings and data analysis. Patch-clamp recordings were obtained in acutely prepared coronal hypothalamic slices containing the SON, as previously described (32). Hippocampal slices were also prepared from the same rats for the patch-clamp recording in DGCCs. Briefly, rats were anesthetized with ketamine and xylazine (80 mg/kg and 12 mg/kg ip, respectively), decapitated,
and their brains rapidly extracted. Slices were perfused with artificial cerebrospinal fluid (aCSF) (in mM): 126 NaCl; 2.5 KCl; 1 MgSO4; 26 NaHCO3; 1.25 NaH2PO4; 20 glucose; 0.4 ascorbic acid; 1 CaCl2; 2 pyruvic acid; pH was 7.3–7.4, saturated with 95% O2-5% CO2.

Recordings were obtained at room temperature from 121 slices of 56 rats, using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Current and voltage output were filtered at 2 kHz and digitized at 10 kHz (Digitida 1322A, pClamp 9 software Axon Instruments). Patch pipettes were filled with a high Cl−-containing solution (in mM): KCl 140, HEPES 10, Mg2+-ATP 5, MgCl2 0.9, and EGTA 10. For current-clamp experiments, patch pipettes were filled with a more physiological concentration of Cl− (in mM): 130 K-glucuronate, 10 KCl, 10 HEPES, 5 MgATP, and 10 EGTA.

Spontaneous inhibitory postsynaptic currents (sIPSCs, recorded at −70 mV), were detected and analyzed using Mini Analysis (Synaptosoft, Decatur, GA). The currents were recorded in the presence of 6-cyano-7-nitroquinoline-2,3-dione (100 nM), were detected and analyzed using Mini Analysis (SynaptoSoft, Decatur, GA). The currents were recorded in the presence of 6-cyano-7-nitroquinoline-2,3-dione (100 nM) to isolate IPSCs. The holding current (Iholding) and root mean square (RMS) noise were measured in 50-ms epochs of traces lacking IPSCs, separated by ~800 ms, in periods of control aCSF and in the presence of drugs and additional GABAAR blockers (n = 40 epochs in each case). The GABAAR receptor-mediated tonic current (Itonic) was defined as the difference in Iholding before and after application of GABAAR receptor blocker bicuculline (20 μM) or picrotoxin (300 μM). RMS noise was measured in the same epochs using MiniAnalysis.

To study the effects of Itonic on firing discharge, recordings were performed in the current-clamp mode. Firing discharges (spontaneous or evoked using DC current injection) were recorded in continuous mode. Firing rate was calculated using Mini Analysis, by counting the number of action potentials in 10-s bins, for a period of ~3 min before and after bath application of THIP. Mean values for each condition were then obtained.

Drugs were added to the perfusing aCSF solution at known concentrations. The final concentration of DMSO was less than 0.05%, when used to dissolve drugs. All drugs were purchased from Sigma-Aldrich (St. Louis, MO).

RT-PCR. Using the RNaseq total RNA isolation kit (Qiagen, Valencia, CA), total RNA was extracted from the SON, and the hippocampus was microdissected from 300-μm-thick acute coronal slices, and treated with 10 U of RNase-free DNase 1 (Invitrogen, Carlsbad, CA) for 30 min at 37°C. For the SON, the tissue punches were pooled from three rats. Reverse transcription was performed using the SuperScript III First-Strand Synthesis System (Invitrogen), according to the protocol of the manufacturer. Amplification of cDNAs via PCR was performed using primer pairs designed to amplify the GABAAR α3-subunit (5'-GCC AAA GGA GCT TCC TTA ACC-3' and 5'-ATT GGC TCC CTT TGT TGT AG-3'), γ2-subunit (5'-CCG AAA CCA AGC AAG GAT AA-3' and 5'-GGG CAA GCA GAA GGC GGT AG-3'), and GABAAR β-subunit (5'-GGG GCC ATC CGT TCC AGA CTC AA-3' and 5'-TCC TCT CGT TCC CAC TTG GG-3'), respectively. All primers were synthesized by Bioneer (Daejeon, Korea). Mixture of PCR reaction contained (in μL): 1 of 10 pmol each primer, 12.5 × master mix buffer (Go Taq Green Master Mix, Promega, Fitchburg, WI), 2 dimethyl sulfoxide, and 4 cDNA template. The annealing temperature in the thermal cycler was 60°C, and 30 cycles were performed. Final PCR products were detected by ethidium bromide staining. All of the PCR products were purified using a PCR purification kit (Qiagen) and confirmed by sequencing.

Real-time PCR was performed using StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA) with cDNA from the SON slices and dissected hippocampal dentate gyrus. Primers were designed using Primer Express 2.0 (Applied Biosystems), and synthesized in Cosmo Genetech (Kyonggi-do, Korea) (α3-subunit, 5'-GCC AAT GCA GCT TGA GGA CTT-3' and 5'-GAA TTA GGG TAA GCA TAA CTT CCA A-3', γ2-subunit, 5'-AAC AAA CTT CGG CCC GAC A-3' and 5'-GCA TTC ACT GGA CCA ATG CTG-3', β-subunit, 5'-ACT GCC GCA GTT CAC TAT CAC C-3' and 5'-TGG CCA GCT GTT AGT AAG TTC-3'). The reactions contained 0.7 μL of cDNA template, 0.2 μL of 10 μM in each forward and reverse primer, 0.4 μL of 50 × Rox dye, 10 μL of 2 × SYBR master mix (SYBR Premix Ex Taq: Takara Bio, Shiga, Japan), and 8.5 μL of nuclelease-free water in total 20 μL volumes. Thermal protocol was as follows: a predenaturation at 95°C for 10 s, amplification with 40 cycles of denaturation at 95°C for 5 s, and annealing at 60°C for 25 s, and dissociation stage programmed in the system for melt curve analysis for PCR product specificity. No contamination of the genomic DNA was detected from negative controls by running RT without the reverse transcriptase. The Ct (threshold cycle) values for transcripts were obtained from StepOne Software 2.0 (Applied Biosystems). All samples were run in duplicate and averaged to use for further calculations. The results were analyzed using the 2-ΔΔCt method (24, 41). The relative mRNA expression level was normalized by β-actin. Primer efficiency for each target and reference were calculated using the equation E = 10−(1/ΔCt) (33) to apply the 2-ΔΔCt method.

Statistical analysis. Numerical data are presented as means ± SE. Paired Student’s t-test was used to compare the effects of drug treatment. Repeated-measures analysis of variance (ANOVA-RM), followed by Tukey post hoc tests, were used as needed. Cumulative histograms were compared using Kolmogorov-Smirnov tests.

RESULTS

Selective facilitation of THIP on Itonic over Iphasic in SON MNCs. The δ-subunit-containing GABAAR receptors responsible for Itonic are a preferential target for endogenous neurosteroids in the brain (5, 46). To determine the functional contribution of δ-subunit-containing receptors to Itonic in SON MNCs, we tested the effects of THIP (4,5,6,7-tetrahydroisothiazolo[5,4-c]pyridin-3-ol), a GABAAR receptor agonist preferentially activate δ- over γ-containing GABAAR receptors (1, 6, 13) (Fig. 1). A low concentration of THIP (100 nM) caused no significant change in Iholding and RMS, or in major properties of IPSCs (Fig. 1). However, bath application of 1 μM THIP caused a significant inward shift in Iholding (ΔΔCt 12.24 ± 2.77 pA, n = 11, P < 0.01) and RMS increase (Fig. 1A), an effect that was blocked by the GABAAR receptor blocker bicuculline (BIC) or picrotoxin. In contrast, THIP (1 μM) induced no detectable changes in the frequency, amplitudes, and decay time of IPSCs (Fig. 1B). Despite its selective modulation of Itonic but not Iphasic, THIP (1 μM) significantly attenuated the firing activity of SON MNCs (Fig. 1C).

These results reinforced our hypothesis that GABAAR receptors mediating phasic and tonic inhibition have distinct molecular configuration and that the latter inhibitory modality plays a major role in modulating SON neuronal excitability (32).

DS-2 has no effects on Itonic in SON MNCs. To further verify the functional contribution of δ-subunit-containing receptors in Itonic of SON MNCs, we measured Iholding and RMS changes in response to application of DS-2, which preferentially enhances the interaction of GABA with δ- over γ-containing GABAAR receptors (49).

Bath application of DS-2 (30–100 μM) caused minimal changes in Iholding and RMS in SON MNCs (P > 0.6 in both cases) (Fig. 2A). To determine whether DS-2 acts as a modulator of GABAAR δ-subunit, we tested the effects of DS-2 on Itonic of DGCCs in hippocampal slices, mediated by δ-subunit-
containing receptors (48). DS-2 (30 μM) caused a significant inward shift in \( I_{\text{holding}} \) (15.04 ± 3.75 pA, \( n = 7 \), \( P < 0.01 \)) along with an increase in RMS, effects that were blocked by the GABA\(_A\) receptor blocker bicuculline (BIC) (Fig. 2B). These results suggest that GABA\(_A\)R \( \delta \)-subunits contribute to a much lesser extent to \( I_{\text{tonic}} \) in SON MNCs than they do in DGGCs.

**Inhibition of L-655,708 on \( I_{\text{tonic}} \) in SON MNCs.** In addition to \( \delta \)-subunit-containing GABA\(_A\)R, \( \alpha_2 \)-subunit-containing GABA\(_A\) receptors have been known to mediate \( I_{\text{tonic}} \) in the hippocampus (15, 25). To determine whether this was also the case in SON MNCs, we measured \( I_{\text{holding}} \) and RMS changes during the application of L-655,708, a GABA\(_A\)R \( \alpha_5 \)-subunit selective partial inverse agonist (11, 37).

Bath application of L-655,708 (5 μM) outwardly shifted \( I_{\text{holding}} \) (16.35 ± 3.94 pA, \( n = 7 \)) but had no effect on IPSCs amplitude (control: 237.34 ± 40.08 vs. L-655,708, 188.08 ± 19.38, \( n = 10 \), \( P > 0.1 \)) and frequency (control: 1.48 ± 0.24 vs. L-655,708, 1.38 ± 0.30, \( n = 10 \), \( P > 0.3 \)). To further assess the contribution of GABA\(_A\)R \( \alpha_5 \)-subunit on \( I_{\text{tonic}} \), we tested the effects of L-655,708 in the presence of GABA (3 μM) added in the perfusion solution. In the presence of 3 μM GABA, L-655,708 (5 μM) induced significantly larger outward shifts in \( I_{\text{holding}} \) (16.35 ± 3.94 pA, \( n = 7 \)) than those in normal aCSF (15.04 ± 3.75 pA, \( n = 7 \)) with slight decreases in IPSCs decay time (control: 19.77 ± 1.46 vs. L-655,708, 17.58 ± 1.28, \( n = 10 \), \( P < 0.01 \)) and had no effect on IPSCs amplitude (control: 237.34 ± 40.08 vs. L-655,708, 188.08 ± 19.38, \( n = 10 \), \( P > 0.1 \)). Consistently, L-655,708 caused a decrease in RMS noise in the presence of 3 μM GABA (P < 0.01, compared with normal aCSF) (Fig. 3, A and B).

Interestingly, the L-655,708-sensitive portion of the total \( I_{\text{tonic}} \) uncovered by the additional application of BIC was not...
different in the absence or presence of 3 μM GABA (P > 0.5) (Fig. 3C).

These findings indicate that GABAA,R α5-subunit-containing receptors contribute to Itonic in SON MNCs, both under conditions of low endogenous or elevated ambient GABA concentration.

Facilitation of Itonic by benzodiazepine in SON MNCs. Diazepam enhancement of GABA currents requires GABAA,R α1–3- or α5-subunit associated with the γ2-subunit (4), whereas zolpidem differentiates α5-containing GABAA,R receptors from α1–3-containing receptors by its low sensitivity to the α5-subunit (36, 40, 45). To further verify the involvement of α5-subunit-containing GABAA,R receptors, we determined the sensitivity of Itonic to benzodiazepines in SON MNCs. Diazepam (1 μM) caused a significant inward shift in Iholding (Δ22.06 ± 3.22 pA, n = 7), and increased RMS from 3.20 ±
was omitted (−RT), indicating no genomic DNA contamination (data not shown). The lesser expression of δ-subunit mRNA than γ2- and α5-subunit mRNA was further confirmed by real-time RT-PCR in the SON (Fig. 5B). Furthermore, δ-subunit mRNA expression level in the SON was even less than in hippocampal dentate gyrus (Fig. 5C).

Facilitation of Itonic and Iphasic by pregnane steroids in SON MNCs. To assess neurosteroid modulation on Itonic in SON MNCs, we tested the effects of the pregnane steroids (allopregnanolone and 3α,5α-THDOC) on Iholding and RMS in the neurons (Fig. 6).

Bath application of the allopregnanolone caused a dose-dependent inward shift in Iholding (∆4.73 ± 1.211 pA; n = 7 and ∆37.61 ± 7.77 pA, n = 14, 0.1 and 1 μM allopregnanolone, respectively), an effect blocked by the GABAAR receptor blocker bicuculline or picrotoxin. Along with an increase of Iholding, RMS increased by 0.1 (P < 0.05) and 1 μM allopregnanolone (P < 0.01), respectively (Fig. 6A). Similar effects were observed when another pregnane steroid 3α,5α-THDOC was used. Results are summarized in Fig. 6B. 3α,5α-THDOC (1 μM) increased Iholding (∆34.46 ± 8.90 pA, n = 18) and RMS, respectively (P < 0.01 in both cases).

Consistent with previous reports (7, 14), allopregnanolone also prolonged the decay time of GABAAR receptor-mediated IPSCs (~130% and 280% by 0.1 and 1 μM allopregnanolone, respectively) without any significant change in the frequency of IPSCs. 3α,5α-THDOC (1 μM) also prolonged the decay time of IPSCs (n = 18, P < 0.01) with no changes in IPSC frequency. Major properties of IPSCs before and during the presence of the steroids are summarized in Table 1.

Altogether, these data support that both tonic and phasic GABAAR inhibition are effective targets of neurosteroid modulation in SON MNCs.
Relative contribution of phasic and tonic modality to neurosteroid facilitation of GABA_A inhibition. To determine the relative contribution of Iphasic and Itonic to neurosteroid facilitation of GABA_A inhibition, we estimated and compared the neurosteroid-facilitated charge transfer and mean currents mediated by the two inhibitory modalities. The mean Iphasic was calculated by multiplying the charge transfer of the averaged IPSC (the integrated area under sIPSC) by the sIPSC frequency for the comparison, as previously described (32). Consistent with the previous report, at a frequency of 2.5 Hz, IPSC charge transfer (2.62 ± 0.17 pC, n = 35) resulted in Iphasic of 7.82 ± 0.52 pA in SON MNCs.

Although the neurosteroids increased both Iphasic and Itonic, as shown by the prolonged decay time of IPSCs and inward shift in Iholding, respectively, the overall increase was mainly mediated by Itonic. Indeed, Itonic increase reached ~5 times and ~18 times of the mean Iphasic increase in the presence of 0.1 and 1 µM allopregnanolone, respectively (Fig. 7).

![Fig. 6. Neurosteroids modulation of Itonic in SON neurons. A1: representative example showing that allopregnanolone (ALLO; 1 µM) induced an inward shift in Iholding and increased RMS, which were blocked by the GABA_A receptor antagonist picrotoxin (PIC). IPSCs were truncated for clarity. Mean changes in Iholding and RMS induced by 0.1 µM (n = 7) and 1 µM (n = 14) allopregnanolone are summarized in A2 and A3, respectively. B1: representative example showing that bath application of 3α,5α-THDOC (1 µM) caused a significant inward increase in Iholding of SON neurons. Mean changes in Iholding and RMS (n = 18) are summarized in B2 and B3, respectively. Summarized data are expressed as means ± SE. *P < 0.05, **P < 0.01, and ***P < 0.001, compared with its respective control.

![Fig. 7. Comparison of neurosteroid effect on Itonic and Iphasic. Mean data summarizing Iphasic and Itonic enhanced by allopregnanolone (0.1 µM, n = 7 and 1 µM, n = 14) and 3α,5α-THDOC (1 µM, n = 18). Iphasic increase was determined by Iholding, shift caused by the steroids. Note the much larger Itonic increases than those of Iphasic. Data shown are expressed as means ± SE. *P < 0.05 and ***P < 0.001 compared with Iphasic increase.](http://ajpregu.physiology.org/)

### Table 1. Effects of pregnane steroids on major properties of IPSCs

<table>
<thead>
<tr>
<th>IPSCs property</th>
<th>Frequency, Hz</th>
<th>Amplitude, pA</th>
<th>Weighted τ, ms</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.25 ± 1.49</td>
<td>214.4 ± 40.2</td>
<td>17.44 ± 0.98</td>
<td>7</td>
</tr>
<tr>
<td>ALLO, 0.1 µM</td>
<td>3.00 ± 1.30</td>
<td>199.2 ± 30.8</td>
<td>22.61 ± 1.77†</td>
<td>16</td>
</tr>
<tr>
<td>Control</td>
<td>3.03 ± 0.89</td>
<td>185.6 ± 10.2</td>
<td>18.29 ± 1.37</td>
<td>16</td>
</tr>
<tr>
<td>ALLO, 1 µM</td>
<td>2.41 ± 0.58</td>
<td>163.6 ± 9.08*</td>
<td>49.30 ± 6.72‡</td>
<td>18</td>
</tr>
<tr>
<td>Control</td>
<td>2.84 ± 0.56</td>
<td>211.6 ± 20.2</td>
<td>23.07 ± 1.49</td>
<td>18</td>
</tr>
<tr>
<td>THDOC, 1 µM</td>
<td>2.62 ± 0.46</td>
<td>181.3 ± 12.9†</td>
<td>61.55 ± 9.55‡</td>
<td>18</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. IPSCs, inhibitory postsynaptic currents; ALLO, allopregnanolone; THDOC, 3α,5α-THDOC. *P < 0.05, †P < 0.01, and ‡P < 0.001 before and after drug.
Similar results were observed with 3α,5α-THDOC application. Despite their significant increase to ~220% of control and because of their transient nature and rapid kinetics, the overall increase of mean $I_{\text{phasic}}$ was negligible compared with $I_{\text{tonic}}$ increase in the presence of the steroid. At an average frequency of 2.84 ± 0.56 Hz ($n = 18$), 3α,5α-THDOC increased mean $I_{\text{phasic}}$ by 1.17 ± 0.46 pA, which was much smaller than $I_{\text{tonic}}$ increase (34.46 ± 8.90 pA) ($n = 18$, $P < 0.01$) (Fig. 7).

Thus, our results indicate that most of GABA$_A$ receptor-mediated inhibition (phasic + tonic) is carried by the tonic modality during neurosteroid modulation, as we previously observed under normal basal conditions in SON neurons (32).

**Effects of L-655,708 on $I_{\text{tonic}}$ facilitation by pregnane steroids.** To assess the contribution of GABA$_A$R α$_5$-subunit to neurosteroid modulation of $I_{\text{tonic}}$, we tested whether L-655,708 attenuated $I_{\text{tonic}}$ facilitation by the pregnane steroids. Results are summarized in Fig. 8.

In the presence of L-655,708, allopregnanolone still facilitated $I_{\text{tonic}}$ as shown by increased $I_{\text{holding}}$ and RMS (Fig. 8A). $I_{\text{phasic}}$ was also potentiated by allopregnanolone, as shown by the increased decay time of IPSCs (Figs. 8B). Importantly, L-655,708 attenuated allopregnanolone-induced $I_{\text{holding}}$ increase ($P < 0.01$), while it did not alter the prolongation of IPSCs decay time by the steroid ($P > 0.7$) (Fig. 8C). In the presence of L-655,708, allopregnanolone-induced inward currents were decreased by ~30% compared with those observed in control aCSF.

**DISCUSSION**

The main findings in the present study may be summarized as followed: 1) $I_{\text{tonic}}$ in magnocellular SON neuron is largely mediated by benzodiazepine-sensitive GABA$_A$ receptors containing α$_5$-, β$_1$-, and γ$_2$-subunits; 2) the minor contribution of GABA$_A$R δ subunits to $I_{\text{tonic}}$ likely explains the similar neurosteroid sensitivity of $I_{\text{tonic}}$ and $I_{\text{phasic}}$ in SON neurons; and 3) $I_{\text{tonic}}$ is the prevailing GABA$_A$ inhibitory modality in SON MNCs, both under basal conditions and during neurosteroids facilitation. Taken together, our data provide novel information on neurosteroid modulation in the SON, showing that in addition to the facilitation of $I_{\text{phasic}}$ (8, 14, 23), pregnane neurosteroids also, and perhaps predominantly, modulate $I_{\text{tonic}}$.

**GABA$_A$R δ-subunit and $I_{\text{tonic}}$ in SON MNCs.** The δ-subunit-containing receptors have been known to be ideally suited to mediate a persistent $I_{\text{tonic}}$ (6). Combined with the GABA$_A$R δ-subunit expression (34), our data showing the selective facilitation of THIP on $I_{\text{tonic}}$ but not $I_{\text{phasic}}$ supported the functional presence and selective contribution of GABA$_A$R δ-subunit in $I_{\text{tonic}}$ of SON MNCs. We previously reported similar characteristics of $I_{\text{tonic}}$ in presympathetic paraventricular nucleus (PVN) neurons (30, 31). Despite selective facilitation of $I_{\text{tonic}}$ over $I_{\text{phasic}}$ by 1 μM THIP, the effects of THIP at nanomolar concentrations (10–100 nM) were negligible both in presympathetic PVN neurons and SON MNCs, arguing against a dominant role of GABA$_A$R δ-subunit in mediating $I_{\text{tonic}}$ in these neurons. Furthermore, even 1 μM THIP caused a much smaller increase of $I_{\text{tonic}}$ in SON MNCs than that evoked by 3 μM GABA in the present study (THIP, 12.25 ± 2.77 pA, n = 11 vs. GABA, 46.20 ± 4.71 pA, n = 7, $P < 0.001$) (Figs. 1 and 3, respectively). Given that GABA acts only as a partial agonist on GABA$_A$R con...
containing δ-subunit, which is a preferential target of the “superagonist” THIP at nanomolar range (46), these results suggest a minor role of GABAAR δ-subunit in I_{tonic} of SON MNCs. The notion is in agreement with relatively low expression of the δ-subunit mRNA in the SON (Fig. 5), and no expression of GABAAR α5- and α6-subunits (34) known to mediate I_{tonic} in association with the δ-subunit in DGCCs and CGCs, respectively.

I_{tonic} amplitudes could vary depending on several conditions, including extracellular GABA concentrations in the slice preparations (for review, see Ref. 19). For example, I_{tonic} in DGCCs of male Sprague-Dawley rats (150–200 g) ranges from 2.3 ± 0.3 pA (52) to 72 ± 0.3 pA (27) in normal aCSF. In our slice preparations, I_{tonic} in DGCCs (3.2 ± 0.8 pA, n = 4) was relatively small (data not shown) and reached only 1/3 of that in SON MNCs. However, it is noteworthy that DS-2, a minimal response of I_{tonic} to DS-2 supported a relatively lesser α5-subunit in the SON than in the dentate gyrus (Fig. 5), a condition that shows the occurrence of a subunit mRNA ratio that is unique to the SON (50).

These results support a major role of GABAAR δ-subunit in mediating I_{tonic} in SON MNCs. GABAAR α5- and α6-subunit contribute to I_{tonic} only when ambient GABA concentrations increase but not by endogenous ambient GABA (42), while both α5- and δ-subunit-containing GABAAR mediate I_{tonic} in CA1 neurons (10, 20, 35, 42). The zolpidem-sensitive I_{tonic} possibly mediated by GABAAR α5-subunits have been uncovered only by the increased extracellular GABA concentration in hippocampal interneurons (43) and preganglionic neurons of dorsal motor nucleus of the vagus (18). Therefore, unlike the neurons, our results show that L-655,708 blocked basal I_{tonic} in normal aCSF indicated that endogenous ambient GABA is enough to activate GABAAR α5-subunit-containing receptors in SON MNCs. This notion was supported by the result that L-655,708 inhibited a similar portion of I_{tonic} due to endogenous and raised ambient GABA in SON MNCs. Therefore, α5-subunit-containing GABAAR receptor may be one of the major contributing isoforms mediating I_{tonic} throughout the wide physiological range of ambient GABA concentrations in SON MNCs.

Diazepam enhancement of GABA currents in mature animals requires GABAAR α1-3 or α5-subunit associated with γ2-subunit (4). Therefore, our data showing that diazepam increased the amplitude of I_{tonic} suggest that GABAAR α5 associated with γ2-subunits are involved in the tonic inhibition of SON MNCs. In addition, our results show a zolpidem-induced I_{holding} shift suggest that additional isoforms such as α1-subunit are also involved in I_{tonic} of SON MNCs. Given that zolpidem differentiates α5-containing GABAAR receptors from α1-3-containing receptors by the low sensitivity to α5-subunit (36, 40, 45), the preferential modulation of diazepam over zolpidem on I_{tonic} in line with the premise that α5-subunit-containing GABAAR, among others coupled to βγ2-subunit, dominantly contributes to I_{tonic} in SON MNCs. To confirm the coupling between the subunits in the neurons, future studies using immunoprecipitation or transgenic animals are warranted.

Neurosteroid sensitivity of I_{tonic} in SON MNCs. Our data have suggested the minor role of GABAAR δ-subunit in I_{tonic} facilitation of pregnane steroids in SON MNCs. The δ-subunit-containing receptors have been known to be more sensitive to neurosteroids than their counterparts containing the γ2-subunit (6, 46). Indeed, small basal I_{tonic} predominantly mediated by δ-subunit-containing GABAAR receptors is increased by 3α,5α-THDOC in CA1 neurons (42), while I_{tonic} mediated by another isoform, such as α5-subunit-containing GABAAR receptors with raised ambient GABA, is unaffected by the neurosteroid (20, 46). In this sense, the similar sensitivities of I_{phasic} and I_{tonic} to the pregnane steroids in the present study indicate a major role of γ2-subunit- rather than δ-subunit-containing receptors in I_{tonic} of SON MNCs. It is also consistent with our results showing that endogenous ambient GABA is enough to activate α5-subunit-containing GABAAR receptors in SON MNCs and that the GABAAR δ-subunit was expressed to a much lesser degree than α5- or γ2-subunit. However, we cannot completely exclude the possibility that δ receptors also play a partial role in I_{tonic} facilitation of pregnane steroids.

Although the possibility was argued by our result that THIP facilitated the I_{tonic} but not I_{phasic} in SON MNCs, similar sensitivities of I_{phasic} and I_{tonic} to the steroids raised the possibility that the two modalities are mediated by the same GABAAR receptor isoform. Indeed, GABAAR receptors containing α5- and α6-subunit mediate both I_{phasic} and I_{tonic} in CA1 neurons (51) and pyramidal neurons of neocortex (50), respectively. However, the significant effects of α5-subunit-containing GABAAR receptor inverse agonist L-655,708 on I_{tonic} with minor inhibitory effects on I_{phasic} in the present study support that GABAAR α5-subunits contribute dominantly to I_{tonic} rather than I_{phasic} in SON MNCs. Furthermore, our results showing that L-655,708 inhibited the facilitation of pregnane steroids on I_{tonic} with negligible effects on I_{phasic} support that GABAAR α5-subunits are selective targets of neurosteroid modulation of I_{tonic} but not of I_{phasic} in SON MNCs.

In the present study, I_{tonic} responses to the steroids vary in different SON neurons but could not be classified in two or more groups (data not shown). This may be consistent with our previous report that I_{tonic} is not different in vasopressinergic and oxytocinergic neurons identified by post hoc immunohistochemistry (32). It is noteworthy that marked plasticity in GABAAR receptors occurs in adult oxytocin neurons of the SON during pregnancy (16, 17). I_{phasic} is potentiated by neurosteroids in oxytocin neurons expressing a relatively high α1:α2-subunit mRNA ratio, but relatively insensitive in neurons with a lower α1:α2-subunit mRNA ratio at parturition period (7). Although our data suggest that the major role of α5-subunit in basal I_{tonic} in SON MNCs, an increased contribution of other α-subunit by neurosteroid challenges cannot be ruled out, especially in oxytocin neurons.

Physiological significance of neurosteroid modulation of I_{tonic} in SON MNCs. Our findings indicate that submicromolar to micromolar concentrations of allopregnanolone and THDOC that may occur during parturition (12, 47) facilitate I_{tonic}, as well as prolong IPSCs in SON MNCs. Under basal conditions, I_{tonic}
accounted for more than ~70% of the total GABA\textsubscript{A}R-mediated current in various brain regions, including SON MNCs (29, 31, 32). In the present study, I\textsubscript{tonic} also mediated a major portion of the total GABA\textsubscript{A}A-receptor-mediated current during neurosteroid facilitation, even though the pregnane steroids facilitated both I\textsubscript{phasic} and I\textsubscript{tonic}. Modulation of basal I\textsubscript{tonic}, without necessarily increasing the efficacy of excitatory inputs, can directly affect intrinsic properties and firing output of SON neurons (32). Thus, the GABA\textsubscript{A}A tonic inhibitory modulation is expected to have a major impact on SON neuronal excitability in both basal and elevated neurosteroid levels of the nucleus.

One of the most common physiological conditions associated with elevated steroid hormone levels is pregnancy. During pregnancy, progesterone levels rise ~200-fold, and there are also large increases in the levels of the neuroactive steroids allopregnanolone and THDOC (12). Previous studies reported that the fall in progesterone at term pregnancy precipitates the fall in oxytocin at term pregnancy (33). The present study also showed that GABA\textsubscript{A}A receptors mediating I\textsubscript{tonic} constitute a functionally relevant target for neurosteroid modulation of GABA action in SON MNCs. Taken together, these results lead to the proposal that neurosteroid modulation of I\textsubscript{tonic}, as well as I\textsubscript{phasic} in the SON, could be an important mechanism mediating neuronal function and hormonal output in pregnancy.

In summary, our findings in the present study suggest, in addition to the facilitation of I\textsubscript{phasic}, progesterone neurosteroids, perhaps predominantly, modulate I\textsubscript{tonic} mediated by \alpha\textsubscript{5}- and \gamma\textsubscript{2}-subunit-containing GABA\textsubscript{A} receptors in SON MNCs. The results provide significant insights into the molecular configurations of GABA\textsubscript{A}R underlying I\textsubscript{tonic} and its functional significance in neurosteroid modulation in the magnocellular neurosecretory system.

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Present address of C.-H. Cho: Dept. of Pharmacology, College of Medicine, Seoul National University, Seoul, Republic of Korea.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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