Neurosteroid modulation of benzodiazepine-sensitive GABA\textsubscript{A} tonic inhibition in supraoptic magnocellular neurons

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The magnocellular neurosecretory cells (MNCs) of the supraoptic nucleus (SON) project to the neurohypophysis (2), where they release oxytocin or vasopressin into the bloodstream, and play fundamental roles in reproduction and fluid homeostasis. GABA, through activation of GABA\textsubscript{A} receptors (GABA\textsubscript{A}R), is a major neurotransmitter modulating neuronal excitability in SON (21, 26, 39). In SON MNCs, interactions between neurosteroids and GABA\textsubscript{A}R have attracted particular attention. Oxytocin release from the dendrites 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\textsubscript{A}R underlie persistent tonic inhibitory currents (I\textsubscript{tonic}), as well as conventional inhibitory postsynaptic currents (IPSCs, I\textsubscript{phasic}) in the central nervous system (15, 25, 44). GABA\textsubscript{A}R mediating I\textsubscript{phasic} are activated by brief exposure to a high concentration of the neurotransmitter, while the receptors mediating I\textsubscript{tonic} are activated by low ambient concentration of the transmitter in the extracellular space. I\textsubscript{phasic}, originally known in cerebellar (CGCs) and dentate gyrus (DGCCs) granule cells, is mediated by GABA\textsubscript{A}R containing δ-subunit associated with the α\textsubscript{6}-subunit (22, 28) and the α\textsubscript{4}-subunit (48), respectively. I\textsubscript{tonic}, mediated by δ-subunit-containing GABA\textsubscript{A}R, appears more sensitive to neurosteroids than its synaptic counterpart, I\textsubscript{phasic}, which is mediated by γ\textsubscript{2}-subunit-containing receptors. For example, I\textsubscript{tonic} is selectively enhanced by a low concentration of 3α,5α-THDOC that has no effect upon the kinetics of I\textsubscript{phasic} in DGCCs and CGCs (46).

Facilitation of I\textsubscript{phasic} has been considered the primary mechanism whereby neurosteroids influence neuronal excitability in SON MNCs (8, 14, 23). However, GABA\textsubscript{A}R of possibly different molecular configuration mediate I\textsubscript{tonic}, as well as I\textsubscript{phasic} in SON MNCs (32). Despite the wealth of information available on I\textsubscript{phasic}, no information is available so far on the neurosteroids modulation of I\textsubscript{tonic} in SON MNCs. Even whether the steroid modulation on I\textsubscript{tonic} is present in the neurons is unknown. In this study, we obtained information on the molecular configuration of GABA\textsubscript{A} receptors underlying the steroid modulation of I\textsubscript{tonic} and showed the major role of I\textsubscript{tonic} in pregnant steroids potentiation of GABA\textsubscript{A} 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,

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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 (Digidata 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-nitroquinoxaline-2,3-dione (100 μM) 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 GABA_A blockers (n = 40 epochs in each case). The GABA_A receptor-mediated tonic current (Itonic) was defined as the difference in Iholding before and after application of GABA_A 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 MiniAnalysis, 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 RNeasy 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 GABA_A_R α₅-subunit (5'-AAA GGG GCT ACC TCT CCA AA-3' and 5'-ATT GCC TCC CGT TGT TG-3'), γ₂-subunit (5'-CGG AAA CCA AGC AAG GAT AA-3' and 5'-GAA CAA GCA GAA GGC AGT-3'), and GABA_A_R β-subunit (5'-GCC ATC CGT TCC AGT AA-3' and 5'-TCC TGC TTC GTG-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 x 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 electrophoresis in 1.8% agarose gels with 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’-TCC AAT GCA GCT TGA GGA CCT-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 GGC CCA GTT CAC TAT CAC C-3’ and 5’-TGG CCA GCT GAT GTG AAG TTC-3’; β-actin, 5’-CAA GAT CAT TGC TCC TCC TGA G-3’ and 5’-TCC TGC TGT CTG ATC CAC A-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 nuclease-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/ΔSlope) (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 GABA_A 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 GABA_A receptor agonist preferentially activate δ- over γ-containing GABA_A 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 (ΔIholding = ±2.77 ± 0.14 pA, n = 11, P < 0.01) and RMS increase (Fig. 1A), an effect that was blocked by the GABA_A 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 GABA_A 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 GABA_A 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 GABA_A_R 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 $I_{\text{holding}}$ ($\Delta 15.04 \pm 3.75$ pA, $n = 7$, $P < 0.01$) along with an increase in RMS, effects that were blocked by the GABA$_A$ receptor blocker bicuculline (BIC). Inhibitory postsynaptic currents (IPSCs) were truncated for clarity. Mean changes in $I_{\text{holding}}$ and RMS induced by 0.1 μM and 1 μM THIP are summarized in A2 ($n = 5$) and A3 ($n = 11$), respectively. **$P < 0.01$, compared with its respective controls. B1 and B2: cumulative plots for interevent intervals and IPSCs amplitudes from the same neuron as in A before (control) and during bath application of 1 μM THIP, respectively. B3: averaged IPSCs ($n = 120$ events) obtained from the same neuron as in A before and during bath application of THIP. Note that the IPSCs’ properties were not changed by THIP. The effects of 1 μM THIP on the major characteristics of IPSCs are summarized in the bar graphs (insets). Data shown are expressed as means ± SE ($n = 11$). C1: representative example showing that THIP attenuated neuronal firing activity of SON magnocellular neurosecretory cells (MNCs). C2: Summary data depicting changes in firing discharge induced by THIP ($n = 8$) are shown. **$P < 0.001$, compared with control.

Inhibition of L-655,708 on $I_{\text{tonic}}$ in SON MNCs. In addition to δ-subunit-containing GABA$_A$R, α2-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 α2-subunit selective partial inverse agonist (11, 37).

Bath application of L-655,708 (5 μM) outwardly shifted $I_{\text{holding}}$ ($\Delta 4.35 \pm 0.71$ pA, $n = 10$) and decreased RMS ($P < 0.01$ in both cases) (Fig. 3A) with slight decreases in IPSCs decay time (control: 19.77 ± 1.46 vs. L-655,708, 17.58 ± 1.28, $n = 10$, $P < 0.01$) 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 α5-subunit on $I_{\text{tonic}}$, we tested the effects of L-655,708 in the presence 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 $I_{\text{holding}}$ ($\Delta 16.35 \pm 3.94$ pA, $n = 7$) than those in normal aCSF ($P < 0.01$). Consistently, L-655,708 caused a larger 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...
These findings indicate that GABA<sub>A</sub>R 5-subunit-containing receptors contribute to I<sub>tonic</sub> in SON MNCs, both under conditions of low endogenous or elevated ambient GABA concentration.

Facilitation of I<sub>tonic</sub> by benzodiazepine in SON MNCs. Diazepam enhancement of GABA currents requires GABA<sub>A</sub>R 1–3- or 5-subunit associated with the 2-subunit (4), whereas zolpidem differentiates 5-containing GABA<sub>A</sub>R receptors from 1–3-containing receptors by its low sensitivity to the 5-subunit (36, 40, 45). To further verify the involvement of 5-subunit-containing GABA<sub>A</sub>R receptors, we determined the sensitivity of I<sub>tonic</sub> to benzodiazepines in SON MNCs.

Diazepam (1 μM) caused a significant inward shift in I<sub>holding</sub> (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 I_{tonic} and I_{phasic} by pregane steroids in SON MNCs. To assess neurosteroid modulation on I_{tonic} in SON MNCs, we tested the effects of the Pregnanolone and 3α,5α-THDOC on I_{tonic} and RMS in the neurons (Fig. 6).

Bath application of the allopregnanolone caused a dose-dependent inward shift in I_{tonic} (Δ4.73 ± 1.211 pA, n = 7 and Δ37.61 ± 7.77 pA, n = 14, 0.1 and 1 μM allopregnanolone, respectively), an effect blocked by the GABA_A receptor blocker bicuculline or picrotoxin. Along with an increase of I_{tonic}, RMS increased by 0.1 (P < 0.05) and 1 μM allopregnanolone (P < 0.01), respectively (Fig. 6A). Similar effects were observed when another pregname steroid 3α,5α-THDOC was used. Results are summarized in Fig. 6B. 3α,5α-THDOC (1 μM) increased I_{tonic} (Δ34.46 ± 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 GABA_A 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 GABA_A inhibition are effective targets of neurosteroid modulation in SON MNCs.

Expression of GABA_A α₅- and δ-subunit in SON MNCs. To further confirm the presence of the GABA_A receptor subunits δ, γ₂, and α₅ in the SON, we performed RT-PCR analysis of SON tissue punches. Our results showed the presence of mRNA encoding for three GABA_A receptor subunits, although the δ-subunit mRNA was expressed at a considerably lesser degree than γ₂- and α₅-subunit mRNA (Fig. 5A). As a positive control, the presence of mRNA encoding for GABA_A δ-subunit, and the other two subunits was confirmed in hippocampal tissue punches with the same primer pairs. PCR product was not detected in any of the samples in which reverse transcriptase
Relative contribution of phasic and tonic modality to neurosteroid facilitation of GABA\(_A\) inhibition. To determine the relative contribution of I\(_{\text{phasic}}\) and I\(_{\text{tonic}}\) 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 I\(_{\text{phasic}}\) 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 I\(_{\text{phasic}}\) of 7.82 ± 0.52 pA in SON MNCs.

Although the neurosteroids increased both I\(_{\text{phasic}}\) and I\(_{\text{tonic}}\), as shown by the prolonged decay time of IPSCs and inward shift in I\(_{\text{holding}}\), respectively, the overall increase was mainly mediated by I\(_{\text{tonic}}\). Indeed, I\(_{\text{tonic}}\) increase reached ~5 times and ~18 times of the mean I\(_{\text{phasic}}\) increase in the presence of 0.1 and 1 \(\mu\)M allopregnanolone, respectively (Fig. 7).

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

<table>
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<tr>
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<th>Frequency, Hz</th>
<th>Amplitude, pA</th>
<th>Weighted (\tau), ms</th>
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<tbody>
<tr>
<td>Control</td>
<td>3.25 ± 1.49</td>
<td>214.4 ± 40.2</td>
<td>17.44 ± 0.98</td>
</tr>
<tr>
<td>ALLO, 0.1 (\mu)M</td>
<td>3.00 ± 1.30</td>
<td>199.2 ± 30.8</td>
<td>22.61 ± 1.77†</td>
</tr>
<tr>
<td>Control</td>
<td>3.03 ± 0.89</td>
<td>185.6 ± 10.2</td>
<td>18.29 ± 1.37</td>
</tr>
<tr>
<td>ALLO, 1 (\mu)M</td>
<td>2.41 ± 0.58</td>
<td>163.6 ± 9.08*</td>
<td>49.30 ± 6.72‡</td>
</tr>
<tr>
<td>Control</td>
<td>2.84 ± 0.56</td>
<td>211.6 ± 20.2</td>
<td>23.07 ± 1.49</td>
</tr>
<tr>
<td>THDOC, 1 (\mu)M</td>
<td>2.62 ± 0.46</td>
<td>181.3 ± 12.91</td>
<td>61.55 ± 9.55‡</td>
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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 Iphasic was negligible compared with Itonic increase in the presence of the steroid. At an average frequency of 2.84 ± 0.56 Hz (n = 18), 3α,5α-THDOC increased mean Iphasic by 1.17 ± 0.46 pA, which was much smaller than Itonic increase (34.46 ± 8.90 pA) (n = 18, P < 0.01) (Fig. 7).

Thus, our results indicate that most of GABAAR 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 Itonic facilitation by pregnane steroids. To assess the contribution of GABAAR α5-subunit to neurosteroid modulation of Itonic, we tested whether L-655,708 attenuated Itonic facilitation by the pregnane steroids. Results are summarized in Fig. 8.

In the presence of L-655,708, allopregnanolone still facilitated Itonic as shown by increased Iholding and RMS (Fig. 8A). Iphasic was also potentiated by allopregnanolone, as shown by the increased decay time of IPSCs (Figs. 8B). Importantly, L-655,708 attenuated allopregnanolone-induced Iholding 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 follows: 1) Itonic in magnocellular SON neuron is largely mediated by benzodiazepine-sensitive GABAAR receptors containing α5-, β-, and γ2-subunits; 2) the minor contribution of GABAAR δ subunits to Itonic likely explains the similar neurosteroid sensitivity of Itonic and Iphasic in SON neurons; and 3) Itonic is the prevailing GABAAR 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 Iphasic (8, 14, 23), pregnane neurosteroids also, and perhaps predominantly, modulate Itonic.

GABAAR δ-subunit and Itonic in SON MNCs. The δ-subunit-containing receptors have been known to be ideally suited to mediate a persistent Itonic (6). Combined with the GABAAR δ-subunit expression (34), our data showing the selective facilitation of THIP on Itonic but not Iphasic supported the functional presence and selective contribution of GABAAR δ-subunit in Itonic of SON MNCs. We previously reported similar characteristics of Itonic in presympathetic paraventricular nucleus (PVN) neurons (30, 31).

Despite selective facilitation of Itonic over Iphasic 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 GABAAR δ-subunit in mediating Itonic in these neurons. Furthermore, even 1 μM THIP caused a much smaller increase of Itonic 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 GABAAR con-

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**Fig. 8.** Effects of L-655,708 on the steroid facilitation of Itonic. **A**: representative example showing that bath application of ALLO (1 μM) caused a significant inward shift in Iholding in the presence of L-655,708 (top). IPSCs were truncated for clarity. Bottom: Mean changes in Iholding (left) and RMS (right) induced by allopregnanolone are summarized (n = 9). **P < 0.01** compared with its control. **B**: averaged IPSCs (n = 120 events) obtained from the same neuron as in A before and during bath application of allopregnanolone in the presence of L-655,708. The effects of allopregnanolone on the decay time constant of IPSCs are summarized in the bar graphs (insets). Data shown are means ± SE (n = 9). **C**: effects of allopregnanolone on Iholding and the decay time constant of IPSCs in the absence (n = 14) and presence (n = 9) of L-655,708 are summarized. Note that L-655,708 significantly reduced the allopregnanolone-induced Iholding increase, but not the delay of IPSCs decay time by allopregnanolone. **P < 0.01** compared with its control.
taining δ-subunit, which is a preferential target of the “super-
agonist” THIP at nanomolar range (46), these results suggest a
minor role of GABAAR δ-subunit in I\textsubscript{ionic} 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\textsubscript{α5-} and α\textsubscript{δ}-subunits (34) known to mediate I\textsubscript{ionic} in
association with the δ-subunit in DGGCs and CGCs,
respectively.

I\textsubscript{ionic} amplitudes could vary depending on several conditions,
including extracellular GABA concentrations in the slice pre-
parations (for review, see Ref. 19). For example, I\textsubscript{ionic} in
DGGCs 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\textsubscript{ionic} in DGGCs (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
relative selective GABAAR δ-subunit modulator, significantly
potentiates I\textsubscript{ionic} in DGGCs mediated by the δ-subunit (48), but
causd minimal changes in SON MNCs in the same recording
conditions (Fig. 2). Combined with lesser expression of the
δ-subunit in the SON than in the dentate gyrus (Fig. 5), a
minimal response of I\textsubscript{ionic} to DS-2 supported a relatively lesser
role of GABAAR δ-subunit in I\textsubscript{ionic} of SON MNCs than in
DGGCs.

GABAAR α\textsubscript{5}-subunit and I\textsubscript{ionic} of SON MNCs. Our results show that L-655,708, a GABAAR α\textsubscript{5}-subunit inverse agonist,
inhibited I\textsubscript{ionic} under conditions of both endogenous and raised
ambient GABA in SON MNCs by ~35%, close to the
maximal effect of L-655,708 on recombinant receptors (3, 38).
These results support a major role of GABAAR α\textsubscript{5}-subunit in
mediating I\textsubscript{ionic} in SON MNCs. GABAAR α\textsubscript{5}-subunit contrib-
utes to I\textsubscript{ionic} only when ambient GABA concentrations increase
but not by endogenous ambient GABA (42), while both α\textsubscript{5}-
subunit- and δ-subunit-containing GABAAR mediate I\textsubscript{phasic}
in CA1 neurons (10, 20, 35, 42). The zolpidem-sensitive I\textsubscript{ionic}
possibly mediated by GABAAR α\textsubscript{5}-subunits have also been un-
covered only by the increased extracellular GABA concen-
tration in hippocampal interneurons (43) and preganglionic
neurons of dorsal motor nucleus of the vagus (18). Therefore,
unlike the neurons, our results showing L-655,708 blocked
basal I\textsubscript{ionic} in normal aCSF indicated that endogenous ambient
GABA is enough to activate GABAAR α\textsubscript{5}-subunit-containing
receptors in SON MNCs. This notion was supported by the
result that L-655,708 inhibited a similar portion of I\textsubscript{ionic} due to
endogenous and raised ambient GABA in SON MNCs. There-
fore, α\textsubscript{5}-subunit-containing GABAAR receptor may be one of
the major contributing isofoms mediating I\textsubscript{ionic} throughout
the wide physiological range of ambient GABA concentrations in
SON MNCs.

Diazepam enhancement of GABA currents in mature ani-
imals requires GABAAR α\textsubscript{1-3} or α\textsubscript{δ}-subunit associated with
γ\textsubscript{2}-subunit (4). Therefore, our data showing that diazepam
increased the amplitude of I\textsubscript{ionic} suggest that GABAAR α\textsubscript{5}
associated with γ\textsubscript{2}-subunits are involved in the tonic inhibition
of SON MNCs. In addition, our results showing a zolpidem-
induced I\textsubscript{holding} shift suggest that additional isofoms such as
α\textsubscript{1}-subunit are also involved in I\textsubscript{ionic} of SON MNCs. Given that
zolpidem differentiates α\textsubscript{5}-containing GABAAR receptors from
α\textsubscript{1-3}-containing receptors by the low sensitivity to α\textsubscript{5}-subunit
(36, 40, 45), the preferential modulation of diazepam over
zolpidem on I\textsubscript{ionic} is in line with the premise that α\textsubscript{5}-subunit-
containing GABAAR, among others coupled to βγ\textsubscript{2}-subunit,
dominantly contributes to I\textsubscript{ionic} in SON MNCs. To confirm the
coupling between the subunits in the neurons, future studies
using immunoprecipitation or transgenic animals are war-
anted.

Neurosteroid sensitivity of I\textsubscript{ionic} in SON MNCs. Our data have
suggested the minor role of GABAAR δ-subunit in I\textsubscript{ionic} 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 γ\textsubscript{2}-subunit
(6, 46). Indeed, small basal I\textsubscript{ionic} predominantly mediated by
δ-subunit-containing GABAAR receptors is increased by α\textsubscript{3-5α-}
THDOC in CA1 neurons (42), while I\textsubscript{ionic} mediated by another
isofom, such as α\textsubscript{5}-subunit-containing GABAAR receptors with
raised ambient GABA, is unaffected by the neurosteroid (20,
46). In this sense, the similar sensitivities of I\textsubscript{phasic} and I\textsubscript{ionic}
to the pregnane steroids in the present study indicate a major role
of γ\textsubscript{2}-subunit- rather than δ-subunit-containing receptors in
I\textsubscript{ionic} of SON MNCs. It is also consistent with our results
showing that endogenous ambient GABA is enough to activate
α\textsubscript{5}-subunit-containing GABAAR receptors in SON MNCs and
that the GABAAR δ-subunit was expressed to a much lesser
degree than α\textsubscript{5} or γ\textsubscript{2}-subunit. However, we cannot completely
exclude the possibility that δ receptors also play a partial role
in I\textsubscript{ionic} facilitation of pregnane steroids.

Although the possibility was argued by our result that THIP
facilitated the I\textsubscript{ionic} but not I\textsubscript{phasic} in SON MNCs, similar
sensitivities of I\textsubscript{phasic} and I\textsubscript{ionic} to the steroids raised the possi-
bility that the two modalities are mediated by the same
GABAAR receptor isoform. Indeed, GABAAR receptors containing
α\textsubscript{5}- and α\textsubscript{δ}-subunit mediate both I\textsubscript{phasic} and I\textsubscript{ionic} in CA1
neurons (51) and pyramidal neurons of neocortex (50), respec-
tively. However, the significant effects of α\textsubscript{5}-subunit-contain-
ing GABAAR receptor inverse agonist L-655,708 on I\textsubscript{ionic}
with minor inhibitory effects on I\textsubscript{phasic} in the present study support
that GABAAR α\textsubscript{5}-subunits contribute dominantly to I\textsubscript{ionic}
rather than I\textsubscript{phasic} in SON MNCs. Furthermore, our results
showing that L-655,708 inhibited the facilitation of pregnane
steroids on I\textsubscript{ionic} with negligible effects on I\textsubscript{phasic} support that
GABAAR α\textsubscript{5}-subunits are selective targets of neurosteroid modulation of I\textsubscript{ionic} but not of I\textsubscript{phasic} in SON MNCs.

In the present study, I\textsubscript{ionic} 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\textsubscript{ionic} is not different in vasopressinergic
and oxytocinergic neurons identified by post hoc immunohis-
tochemistry (32). It is noteworthy that marked plasticity in
GABAAR receptors occurs in adult oxytocin neurons of the SON
during pregnancy (16, 17). I\textsubscript{phasic} is potentiated by neuro-
steroids in oxytocin neurons expressing a relatively high α\textsubscript{1:α2-
subunit mRNA ratio, but relatively insensitive in neurons with a
lower α\textsubscript{1:α2-subunit mRNA ratio at parturition period (7).
Although our data suggest the major role of α\textsubscript{5}-subunit in
basal I\textsubscript{ionic} 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\textsubscript{ionic} in SON MNCs. Our findings indicate that submicromolar to
micromolar concentrations of allopregnanolone and THDOC that
may occur during parturition (12, 47) facilitate I\textsubscript{ionic} as well as
prolong IPSCs in SON MNCs. Under basal conditions, I\textsubscript{ionic}
accounted for more than ~70% of the total GABA_AR-mediated current in various brain regions, including SON MNCs (29, 31, 32). In the present study, I_{tonic} also mediated a major portion of the total GABA_A-receptor mediated current during neurosteroid facilitation, even though the pregnane steroids facilitated both I_{phasic} and I_{tonic}. Modulation of basal I_{tonic}, without necessarily increasing the efficacy of excitatory inputs, can directly affect intrinsic properties and firing output of SON neurons (32). Thus, the GABA_A tonic inhibitory modality 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 large increases in the levels of the neuroactive steroids pregnane neurosteroids, without necessarily increasing the efficacy of excitatory inputs, can directly affect intrinsic properties and firing output of SON neurons (32). Thus, the GABA_A tonic inhibitory modality is expected to have a major impact on SON neuronal excitability in both basal and elevated neurosteroid levels of the nucleus.

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In summary, our findings in the present study suggest, in addition to the facilitation of I_{phasic} pregnane neurosteroids, perhaps predominantly, modulate I_{tonic} mediated by α5- and γ2-subunit-containing GABA_A receptors in SON MNCs. The results provide significant insights into the molecular configurations of GABA_AR underlying I_{tonic} and its functional significance in neurosteroid modulation in the magnocellular neurosecretory system.

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DISCLOSURES

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

REFERENCES

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