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

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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 (3\(\alpha\),5\(\alpha\)-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 post synaptic 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{tonic}, originally known in cerebellar (CGCs) and dentate gyrus (DGGCs) granule cells, is mediated by GABA\textsubscript{A}R containing \(\delta\)-subunit associated with the \(\alpha_6\)-subunit (22, 28) and the \(\alpha_4\)-subunit (48), respectively. I\textsubscript{tonic}, mediated by \(\delta\)-subunit-containing GABA\textsubscript{A}R, appears more sensitive to neurosteroids than its synaptic counterpart, I\textsubscript{phasic}, which is mediated by \(\gamma_2\)-subunit-containing receptors. For example, I\textsubscript{tonic} is selectively enhanced by a low concentration of 3\(\alpha\),5\(\alpha\)-THDOC that has no effect upon the kinetics of I\textsubscript{phasic} in DGGCs 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 DGGCs. 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, 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-gluconate, 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 (Synapstoposoft, Decatur, GA). The currents were recorded in the presence of 6-cyano-7-nitroquinoline-2,3-dione (100 μM) to isolate IPSCs. The holding current (Iholding) and resting membrane potential (RMP) 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%, and after bath application of THIP. Mean values for each condition were then obtained.

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 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 (ΔIholding = 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). 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 GABAAR receptors (49).
containing receptors (48). DS-2 (30 μM) caused a significant inward shift in \( I_{\text{holding}} \) (Δ15.04 ± 3.75 pA, \( n = 7, P < 0.01 \)) along with an increase in RMS, effects that were blocked by the GABA\(_A\) receptor blocker BIC (Fig. 2B).

These results suggest that GABA\(_A\)R δ-subunits contribute to a much lesser extent to \( I_{\text{tonic}} \) in SON MNCs than they do in DGCCs.

**Inhibition of L-655,708 on \( I_{\text{tonic}} \) in SON MNCs.** In addition to δ-subunit-containing GABA\(_A\)R, α\( _2 \)-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 α\( _5 \)-subunit selective partial inverse agonist (11, 37).

Bath application of L-655,708 (5 μM) outwardly shifted \( I_{\text{holding}} \) (Δ4.35 ± 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, \( n = 10, P > 0.1 \)) 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 α\( _5 \)-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 \( I_{\text{holding}} \) (Δ16.35 ± 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
different in the absence or presence of 3 μM GABA (P > 0.5) (Fig. 3C).

These findings indicate that GABAAR 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 GABAAR α1-3- or α5-subunit associated with the γ2-subunit (4), whereas zolpidem differentiates α5-containing GABAAR 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 GABAAR receptors, we determined the sensitivity of Itonic to benzodiazepines in SON MNCs.

Diazepam (1 μM) caused a significant inward shift in Iholding ($22.06 \pm 3.22$ pA, n = 7), and increased RMS from 3.20...
was confirmed in hippocampal tissue. The presence of diazepam (0.16 pA to 3.49 pA, n = 9) are summarized. Data shown are expressed as means ± SE. **P < 0.05.

Expression of GABAAR α3- and δ-subunit in SON MNCs. To further confirm the presence of the GABAAR 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 all three GABAAR 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 GABAAR 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 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 dosedependent 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.

Fig. 4. Effects of benzodiazepines on ITonic in SON neurons. A: representative current traces showing the outward shift in Iholding by the GABAAR receptor antagonist BIC in the presence of zolpidem (0.5 μM; top) and diazepam (1 μM; bottom) in a SON neuron, respectively. IPSCs were truncated for clarity. Note that the outward shift in Iholding by BIC is much smaller in the presence of zolpidem than in the presence of diazepam. B: amplitudes of Itonic in the presence of zolpidem (0.5 μM), diazepam (3 μM zolpidem, DZP) (n = 4), and 1 μM diazepam (DZP) (n = 9) are summarized. Data shown are expressed as means ± SE. **P < 0.05.

Fig. 5. RT-PCR analysis of GABAAR α3-, γ2-, and δ-subunit expression in SON. A: RT-PCR analysis showed the expression of GABAAR α3-, γ2-, and δ-subunit mRNA in the SON, while minimal PCR product was detected with δ-subunit-specific primer pair. As a positive control, the expression of α3-, γ2-, and δ-subunit mRNA was confirmed in the hippocampus with the same primer pairs. B: real-time RT-PCR summary showed the relative expression of GABAAR δ-, α3-, and γ2-subunit mRNA in the SON. Data shown are expressed as means ± SE (n = 2). The relative expression of α3- and γ2- was normalized to the level detected for δ-subunit. C: relative expression of GABAAR δ-subunit mRNA was compared in the SON (n = 2) and dentate gyrus (n = 3). The data were normalized to the level detected in the SON. Data shown are expressed as means ± SE. ***P < 0.001.
Relative contribution of phasic and tonic modality to neurosteroid facilitation of GABA_A inhibition. To determine the relative contribution of I_phasic and I_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_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 nC, n = 35) resulted in I_phasic of 7.82 ± 0.52 pA in SON MNCs.

Fig. 6. Neurosteroids modulation of I_tonic in SON neurons. A1: representative example showing that allopregnanolone (ALLO; 1 μM) induced an inward shift in I_holding and increased RMS, which were blocked by the GABA_A receptor antagonist picrotoxin (PIC). IPSCs were truncated for clarity. Mean changes in I_holding 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 I_holding of SON neurons. Mean changes in I_holding 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.

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

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>16</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>16</td>
</tr>
<tr>
<td>THDOC, 1 μM</td>
<td>2.62 ± 0.46</td>
<td>181.3 ± 12.91</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.

Fig. 7. Comparison of neurosteroid effect on I_tonic and I_phasic. Mean data summarizing I_phasic and I_tonic enhanced by allopregnanolone (0.1 μM, n = 7 and 1 μM, n = 14) and 3α,5α-THDOC (1 μM, n = 18). I_phasic increase were determined by the difference of mean currents in the absence and presence of the steroids. The mean phasic current was calculated by multiplying the charge transfer by the IPSC frequency. I_tonic increase was determined by I_holding shift caused by the steroids. Note the much larger I_tonic increases than those of I_phasic. Data shown are expressed as means ± SE. *P < 0.05 and ***P < 0.001, compared with I_phasic increase.
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 \( \alpha_5 \)-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 \( \alpha_5 \)-, \( \beta_1 \)-, and \( \gamma_2 \)-subunits; 2) the minor contribution of GABA\( _A \)R \( \delta \)-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 \( \delta \)-subunit and \( I_{\text{tonic}} \) in SON MNCs. The \( \delta \)-subunit-containing receptors have been known to be ideally suited to mediate a persistent \( I_{\text{tonic}} \) (6). Combined with the GABA\( _A \)R \( \delta \)-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 \( \delta \)-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 \( \mu \)M 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 \( \delta \)-subunit in mediating \( I_{\text{tonic}} \) in these neurons. Furthermore, even 1 \( \mu \)M THIP caused a much smaller increase of \( I_{\text{tonic}} \) in SON MNCs than that evoked by 3 \( \mu \)M 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-
neurosteroid modulation of I\text{ tonic} in the son

containing \( \delta \)-subunit, which is a preferential target of the “superagonist” THIP at nanomolar range (46), these results suggest a minor role of GAB\( A_{\alpha R} \) \( \delta \)-subunit in I\text{ tonic} of SON MNCs. The notion is in agreement with relatively low expression of the \( \delta \)-subunit mRNA in the SON (Fig. 5), and no expression of GAB\( A_{\alpha R} \) \( \alpha_\alpha \) and \( \alpha_\delta \)-subunits (34) known to mediate I\text{ tonic} in association with the \( \delta \)-subunit in DGGCs and CGCs, respectively.

I\text{ tonic} amplitudes could vary depending on several conditions, including extracellular GABA concentrations in the slice preparations (for review, see Ref. 19). For example, I\text{ tonic} 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\text{ tonic} in DGGCs (3.2 ± 0.8 pA, \( n = 4 \)) was relatively small (data not shown) and reached only \( \sim 1/3 \) of that in SON MNCs. However, it is noteworthy that DS-2, a minimal response of I\text{ tonic} to DS-2 supported a relatively lesser expression of the \( \delta \)-subunit in SON MNCs. Combined with lesser expression of the ambient GABA levels 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 GAB\( A_{\alpha R} \) \( \delta \)-subunit in mediating I\text{ tonic} in SON MNCs. GAB\( A_{\alpha R} \) \( \alpha_\delta \)-subunit contributes to I\text{ tonic} only when ambient GABA concentrations increase but not by endogenous ambient GABA (42), while both \( \alpha_5 \)-subunit- and \( \delta \)-subunit-containing GAB\( A_{\alpha R} \) mediate I\text{ tonic} in CA1 neurons (10, 20, 35, 42). The zolpidem-sensitive I\text{ tonic} possibly mediated by GAB\( A_{\alpha R} \) \( \alpha_\delta \)-subunits have also 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 showing L-655,708 blocked basal I\text{ tonic} in normal aCSF indicated that endogenous ambient GABA is enough to activate GAB\( A_{\alpha R} \) \( \alpha_\delta \)-subunit-containing receptors in SON MNCs. This notion was supported by the result that L-655,708 inhibited a similar portion of I\text{ tonic} due to endogenous and raised ambient GABA in SON MNCs. Therefore, \( \alpha_5 \)-subunit-containing GAB\( A_{\alpha} \) receptor may be one of the major contributing isoforms mediating I\text{ tonic} throughout the wide physiological range of ambient GABA concentrations in SON MNCs.

Diazepam enhancement of GABA currents in mature animals requires GAB\( A_{\alpha R} \) \( \alpha_{1-3} \) or \( \alpha_\delta \)-subunit associated with \( \gamma_2 \)-subunit (4). Therefore, our data showing that diazepam increased the amplitude of I\text{ tonic} suggest that GAB\( A_{\alpha R} \) \( \alpha_5 \) associated with \( \gamma_2 \)-subunits are involved in the tonic inhibition of SON MNCs. In addition, our results showing a zolpidem-induced I\text{ holding} shift suggest that additional isoforms such as \( \alpha_\delta \)-subunit are also involved in I\text{ tonic} of SON MNCs. Given that zolpidem differentiates \( \alpha_5 \)-containing GAB\( A_{\alpha} \) receptors from \( \alpha_{1-3} \)-containing receptors by the low sensitivity to \( \alpha_\delta \)-subunit (36, 40, 45), the preferential modulation of diazepam over zolpidem on I\text{ tonic} is in line with the premise that \( \alpha_5 \)-subunit-containing GAB\( A_{\alpha R} \), among others coupled to \( \beta_\gamma \)-subunit, dominantly contributes to I\text{ 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\text{ tonic} in SON MNCs.** Our data have suggested the minor role of GAB\( A_{\alpha R} \) \( \delta \)-subunit in I\text{ tonic} facilitation of pregnane steroids in SON MNCs. The \( \delta \)-subunit-containing receptors have been known to be more sensitive to neurosteroids than their counterparts containing the \( \gamma_2 \)-subunit (6, 46). Indeed, small basal I\text{ tonic} predominantly mediated by \( \delta \)-subunit-containing GAB\( A_{\alpha R} \) receptors is increased by \( \alpha_5 \)- or \( \alpha_\delta \)-subunit inverse agonist, such as THIP in CA1 neurons (42), while I\text{ tonic} mediated by another isoform, such as \( \alpha_5 \)-subunit-containing GAB\( A_{\alpha R} \) receptors with raised ambient GABA, is unaffected by the neurosteroid (20, 46). In this sense, the similar sensitivities of I\text{ phasic} and I\text{ tonic} to the pregnane steroids in the present study indicate a major role of \( \gamma_2 \)-subunit- rather than \( \delta \)-subunit-containing receptors in I\text{ tonic} of SON MNCs. It is also consistent with our results showing that endogenous ambient GABA is enough to activate \( \alpha_5 \)-subunit-containing GAB\( A_{\alpha R} \) receptors in SON MNCs and that the GAB\( A_{\alpha R} \) \( \delta \)-subunit was expressed to a much lesser degree than \( \alpha_\delta \) or \( \gamma_2 \)-subunit. However, we cannot completely exclude the possibility that \( \delta \) receptors also play a partial role in I\text{ tonic} facilitation of pregnane steroids.

Although the possibility was argued by our result that THIP facilitated the I\text{ tonic} but not I\text{ phasic} in SON MNCs, similar sensitivities of I\text{ phasic} and I\text{ tonic} to the steroids raised the possibility that the two modalities are mediated by the same GAB\( A_{\alpha} \) receptor isoform. Indeed, GAB\( A_{\alpha} \) receptors containing \( \alpha_5 \) and \( \alpha_\delta \)-subunit mediate both I\text{ phasic} and I\text{ tonic} in CA1 neurons (51) and pyramidal neurons of neocortex (50), respectively. However, the significant effects of \( \alpha_5 \)-subunit-containing GAB\( A_{\alpha} \) receptor inverse agonist L-655,708 on I\text{ tonic} with minor inhibitory effects on I\text{ phasic} in the present study support that GAB\( A_{\alpha R} \) \( \alpha_5 \)-subunits contribute dominantly to I\text{ tonic} rather than I\text{ phasic} in SON MNCs. Furthermore, our results showing that L-655,708 inhibited the facilitation of pregnane steroids on I\text{ tonic} with negligible effects on I\text{ phasic} support that GAB\( A_{\alpha R} \) \( \alpha_5 \)-subunits are selective targets of neurosteroid modulation of I\text{ tonic} but not of I\text{ phasic} in SON MNCs.

In the present study, I\text{ 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\text{ tonic} is not different in vasopressinergic and oxytocinergic neurons identified by post hoc immunohistochemistry (32). It is noteworthy that marked plasticity in GAB\( A_{\alpha} \) receptors occurs in adult oxytocin neurons of the SON during pregnancy (16, 17). I\text{ phasic} is potentiated by neurosteroids in oxytocin neurons expressing a relatively high \( \alpha_1 \); \( \alpha_2 \)-subunit mRNA ratio, but relatively insensitive in neurons with a lower \( \alpha_1 \); \( \alpha_2 \)-subunit mRNA ratio at postparturition period (7). Although our data suggest the major role of \( \alpha_5 \)-subunit in basal I\text{ tonic} in SON MNCs, an increased contribution of other \( \alpha \)-subunit by neurosteroid challenges cannot be ruled out, especially in oxytocin neurons.

**Physiological significance of neurosteroid modulation of I\text{ 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\text{ tonic} in SON MNCs. Our data suggest that neurosteroids differentially modulate I\text{ tonic} in SON MNCs and that GAB\( A_{\alpha} \) \( \delta \)-subunit was expressed to a much lesser degree than \( \alpha_\delta \) or \( \gamma_2 \)-subunit. However, we cannot completely exclude the possibility that \( \delta \) receptors also play a partial role in I\text{ tonic} facilitation of pregnane steroids.
accounted for more than ~70% of the total GABA\(_A\)-R-mediated current in various brain regions, including SON MNCs (29, 31, 32). In the present study, \(I_{\text{ionic}}\) also mediated a major portion of the total GABA\(_A\)-receptor mediated current during neurosteroid facilitation, even though the pregnane steroids facilitated both \(I_{\text{phasic}}\) and \(I_{\text{ionic}}\). Modulation of basal \(I_{\text{ionic}}\), neurosteroid facilitation, even though the pregnane steroids neurosteroid modulation of \(I_{\text{tonic}}\) as well as \(I_{\text{phasic}}\) in the SON, MNCs. Taken together, these results lead to the proposal that target for neurosteroid modulation of GABA action in SON perhaps predominantly, modulate \(I_{\text{tonic}}\) mediated by ratios of GABA\(_A\)-receptor underlying \(I_{\text{tonic}}\) 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.

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