Dual mechanisms diminishing tonic $\text{GABA}_A$ inhibition of dentate gyrus granule cells in Noda epileptic rats

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Running title: Attenuated $I_{\text{tonic}}$ of DGGCs in NER

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Abstract

The Noda epileptic rat (NER), a Wistar colony mutant, spontaneously has tonic–clonic convulsions with paroxysmal discharges. In the present study, we measured phasic ($I_{\text{phasic}}$) and tonic γ-aminobutyric acid A (GABA$_A$) current ($I_{\text{tonic}}$) in NER hippocampal dentate gyrus granule cells (DGGCs) and compared the results with those of normal parent strain Wistar rats (WIS). $I_{\text{tonic}}$, revealed by a bicuculline-induced outward shift in holding current, was significantly smaller in NER than in WIS ($p < 0.01$). The frequency of inhibitory postsynaptic currents (IPSCs) was also significantly lower in NER than in WIS ($p < 0.05$), without significant differences in the IPSC amplitude or decay time between WIS and NER. $I_{\text{tonic}}$ attenuation in NER was further confirmed in the presence of GABA transporter blockers, NO-711 and nipecotic acid, with no difference in neuronal GABA transporter expression between WIS and NER. $I_{\text{tonic}}$ responses to extrasynaptic GABA$_A$ receptor agonists (THIP and DS-2) were significantly reduced in NER compared with WIS ($p < 0.05$). Allopregnanolone caused less $I_{\text{tonic}}$ increase in NER than in WIS, while it prolonged the IPSC decay time to a similar rate in the two groups. Expression of the GABA$_A$ receptor δ subunit was decreased in the dentate gyrus of NER relative to that of WIS. Taken together, our results showed that a combination of attenuated presynaptic GABA release and extrasynaptic GABA$_A$ receptor expression reduced $I_{\text{tonic}}$ amplitude and its sensitivity to neurosteroids, which likely diminishes the gating function of DGGCs and renders NER more susceptible to seizure propagation.

Keywords: GABA$_A$ receptors, Noda epileptic rat, dentate gyrus granule cells
INTRODUCTION

γ-aminobutyric acid A receptors (GABA<sub>A</sub>Rs) mediate the sustained tonic form of inhibition (tonic GABA<sub>A</sub> current, \( I_{\text{tonic}} \)) in addition to conventional fast inhibitory postsynaptic currents (IPSCs; phasic GABA<sub>A</sub> current, \( I_{\text{phasic}} \)) (12, 43), and they consequently have a profound influence on the hippocampal neural circuit. In the forebrain, δ subunit-containing GABA<sub>A</sub>Rs primarily located at perisynaptic and extrasynaptic sites (25, 31, 50, 58) dominantly mediate \( I_{\text{tonic}} \) (21, 51). Conversely, the γ<sub>2</sub> subunit located directly at many GABAergic synapses as well as some extrasynaptic sites (31, 41, 45) plays a major role in \( I_{\text{phasic}} \) (30).

GABA, which is responsible for generating IPSCs, is the main source for \( I_{\text{tonic}} \) under physiological conditions in the hippocampus (15). In experimental epilepsy, the loss of specific subsets of GABAergic interneurons reduces inhibition of dentate gyrus granule cells (DGGCs) (23, 32, 44, 49). Farther away from the releasing boutons, GABA transporters (GATs) decrease the extracellular GABA concentration in a distance- and region-specific manner (9). GAT expression is altered in human and experimental epilepsy (24, 46). Therefore, in addition to reduced expression of the GABA<sub>A</sub>R δ subunit that mediates \( I_{\text{tonic}} \) (28, 36, 42, 60), diminished GABA release and/or enhanced GABA clearance from extracellular space may decrease \( I_{\text{tonic}} \) in epileptic DGGCs. This study tested these possibilities in a spontaneous epilepsy model, as \( I_{\text{tonic}} \) amplitude is maintained or even enhanced by the compensational role of the γ<sub>2</sub> subunit-containing GABA<sub>A</sub>Rs in the DGGCs of chemically-induced status epilepticus (38, 59, 60).

The Noda epileptic rat (NER), a mutant found in an inbred colony of Wistar rats (WIS), has spontaneous tonic–clonic convulsions characterized by the appearance
of high voltage polyspikes in cortical and hippocampal electroencephalography (EEG) in the absence of organic brain lesions (29). Although the cause of the seizures in NER is not clear, tonic–clonic seizures are primarily evoked by activation of forebrain cortico–limbic circuits. No information is available thus far on the role of the hippocampal GABA circuit in NER, whereas the hippocampus has been suggested to play a role in epileptogenesis (17, 33).

Here, we provide novel evidence supporting the attenuation of phasic and tonic GABA_A inhibition of DGGCs in NER, which may increase seizure vulnerability in the spontaneously epileptic rat.
MATERIALS AND METHODS

Experimental animals. NER, supplied by the National BioResource Project – Rat, Kyoto University (Kyoto, Japan), were inbred at the Institute of Laboratory Animals, School of Medicine, Chungnam National University. NER seizure susceptibility was evaluated by audiogenic seizure responses as previously described (20) with slight modification. Briefly, rats were subjected to a bell sound (95 dB and 8 kHz, 30 seconds) weekly starting at 3 weeks of age, and the audiogenic response (ARS) was scored on a scale of 0 to 6 (0 = no response, ARS 1 = neck and forelimb clonus, ARS 2 = wild running, ARS 3 = wild running and jumping, ARS 4 = jumping and clonic convulsion, ARS 5 = generalized tonic convulsion, and ARS 6 = maximal tonic convulsion for more than 5 min). Mean ARS in 8-week-old NER was ~2.5, while no epileptic response was observed in age-matched WIS. WIS were purchased from Samtako Bio (Kyung Gi-Do, Korea). Animals were housed under a 12/12-hour light/dark schedule and allowed free access to food and water until used.

Eight-week-old rats were anesthetized with ketamine and xylazine (80 mg/kg and 12 mg/kg, i.p., respectively) and decapitated 3–5 days after the last audiogenic seizure test. Brains were rapidly extracted for electrophysiological recordings or Western blotting. All animal experimentation was conducted in compliance with the policies of Chungnam National University regarding the use and care of animals and under a license (2009-1-21) issued by the Animal Ethics Committee of Chungnam National University.

Electrophysiological recordings and data analysis. Patch-clamp
recordings were obtained in acutely prepared coronal hippocampal slices (330 µm) from male rats as previously described (22, 34). Briefly, slices were perfused with artificial cerebrospinal fluid (aCSF; in mM: NaCl 126, KCl 2.5, MgSO₄ 1, NaHCO₃ 26, NaH₂PO₄ 1.25, glucose 20, ascorbic acid 0.4, CaCl₂ 1, pyruvic acid 2; pH 7.3-7.4; saturated with 95%O₂–5%CO₂) at a ~3 ml/min flow. Recordings were obtained at 32°C using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Current output was 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).

Spontaneous inhibitory postsynaptic currents (sIPSCs, recorded at −70 mV) were detected and analyzed using MiniAnalysis (Synaptosoft, Decatur, GA). The currents were recorded in the presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM) and 2-amino-5-phosphonovaleric acid (APV, 100 μM) to isolate IPSCs. The holding current (I_holding) and root-mean-square (RMS) noise was measured in 50-ms epochs of traces lacking PSCs separated by ~800 ms by periods of control aCSF and in the presence of drugs and additional GABA_A blockers (n = 40 epochs in each case). I_tonic was defined as the difference between I_holding before and after application of the GABA_A receptor blocker bicuculline (20 μM). RMS noise was measured in the same epochs using MiniAnalysis.

Drugs were added to the perfusing aCSF solution at known concentrations. The final concentration of dimethyl sulfoxide (DMSO) was less than 0.05% when used to dissolve drugs. All drugs except NO-711 (Tocris, UK) were purchased from Sigma-Aldrich (St. Louis, MO).
**Western blotting.** All proteins from dissected dentate gyrus were lysed with 1× passive lysis buffer (Cell Signaling Technology, USA) and quantified using a Coomassie Protein assay kit (BioRad, USA). Approximately 50 µg of protein was electrophoresed on a 10% sodium dodecyl sulfate polyacrylamide gel (SDS–PAGE) and transferred onto nitrocellulose membranes. The blots were blocked with 1× Tris buffered saline (TBS)-Tween 20 containing 3% bovine serum albumin (BSA) + 2% heparan sulfate (HS) for 1 h at room temperature (5% TTBS; Gibco, USA). The blots were then incubated at 4°C with primary antibodies against GABA\(_\alpha\)R \(\delta\) and \(\gamma_2\) subunits and GAT-1 and GAT3 (1:1,000; Millipore, USA) in 5% TTBS, respectively. The next day, the blots were incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:2,000; Santa Cruz Biotechnology, USA). An enhanced chemiluminescence detection kit (ECL; Pierce, USA) was used to visualize antibody binding, and the intensity of the bands was measured using Image J software 1.42q (NIH, USA).

**Statistical analysis.** Numerical data are presented as mean ± standard error of the mean (SEM). Student’s t-tests were used to assess differences between the animal groups.
RESULTS

Electrophysiological recordings were obtained from a total of 114 DGGCs (55 and 59 cells from 22 and 23 heads of WIS and NER, respectively). The GABAR antagonist, bicuculline (BIC, 20 µM), completely blocked spontaneous IPSCs in both WIS and NER (Fig. 1A). In addition to blocking synaptic transmission, BIC outwardly shifted $I_{\text{holding}}$ and reduced RMS, supporting the presence of a sustained GABA$_A$ receptor-mediated tonic current ($I_{\text{tonic}}$) in DGGCs.

Both $I_{\text{phasic}}$ and $I_{\text{tonic}}$ were reduced in NER.

To determine the possible alteration of GABAergic inhibition in DGGCs, we compared $I_{\text{tonic}}$ of NER with that of WIS. There was an approximately 50% decrease in $I_{\text{tonic}}$ amplitude in NER compared with WIS, independent of whole-cell capacitance (WIS, 33.37 ± 1.31 pF vs. NER 31.45 ± 1.13 pF, $n = 30$). As summarized in Fig. 1B, $I_{\text{tonic}}$, indicated by a BIC-induced outward shift in $I_{\text{holding}}$, was significantly smaller in NER (1.74 ± 0.35 pA, $n = 10$) than in WIS (3.36 ± 0.43 pA, $n = 13$; $p < 0.01$). Similarly, the BIC-induced RMS change was less in NER (0.53 ± 0.05 pA, $n = 10$) than in WIS (0.88 ± 0.16, $n = 13$; $p < 0.05$). $I_{\text{tonic}}$ attenuation in NER was further confirmed in the presence of 3 µM GABA (Fig. 1B). $I_{\text{tonic}}$ was significantly smaller in NER (8.86 ± 1.55 pA, $n = 13$) than in WIS (17.32 ± 3.56 pA, $n = 11$; $p < 0.05$) in the presence of 3 µM GABA.

To determine possible differences in synaptic transmission between NER and WIS, a detailed IPSC analysis was conducted, and the results are summarized in Fig. 1C–F. Spontaneous GABA$_A$-mediated IPSCs were observed in all recorded DGGCs. In WIS, GABA$_A$ IPSCs occurred at a mean frequency of 1.59 ± 0.20 Hz, had a mean
amplitude of 51.90 ± 3.12 pA, and decayed with a time course best fitted by a biexponential function ($\tau_{\text{fast}}$: 13.00 ± 1.48 ms; $\tau_{\text{slow}}$: 33.62 ± 2.55 ms) under our recording conditions ($n = 53$). IPSC frequency was significantly lower in NER (1.11 ± 0.13, $n = 55; p < 0.05$) than in with WIS (1.59 ± 0.20, $n = 53$; Fig. 1D), but no significant differences were observed in the IPSC amplitude or decay time constants between the two groups (Fig. 1E and 1F, respectively).

With the notion that the ambient GABA level is under the tight control of presynaptic GABA release in the hippocampus (15), the decreased IPSC frequency and $I_{\text{tonic}}$ amplitude suggests that the lower ambient GABA concentration contributes to $I_{\text{tonic}}$ attenuation in NER.

*Fig. 1 here*

**Diminished $I_{\text{tonic}}$ in the presence of NO-711 in NER**

To further confirm $I_{\text{tonic}}$ attenuation in NER, we measured and compared DGGC $I_{\text{tonic}}$ in the presence of the GAT blocker NO-711 (52) in WIS and NER. Bath application of NO-711 (5 µM) caused a significant inward shift in $I_{\text{holding}}$, an effect that was blocked by the GABA$_A$ receptor blocker BIC in both WIS and NER (Fig. 2A). NO-711 induced significantly less $I_{\text{holding}}$ shift in NER than in WIS. As a result, $I_{\text{tonic}}$ was significantly smaller in NER (14.56 ± 3.41 pA, $n = 7$) than in WIS (28.28 ± 3.53 pA, $n = 7; p < 0.05$) in the presence of NO-711. Consistent with the $I_{\text{tonic}}$ difference, BIC induced a smaller RMS change in NER (1.70 ± 0.34 pA, $n = 7$) than in WIS (2.95 ± 0.41 pA, $n = 7; p < 0.05$; Fig. 2B).

Similar results were obtained with the non-selective GABA transporter blocker,
nipeptic acid (NPA; 100 µM). In NER, BIC caused much less \(I_{\text{holding}}\) shift (WIS, 29.41 ± 8.53 pA, \(n = 6\) vs. NER, 8.19 ± 1.83 pA, \(n = 7\)) and RMS change (WIS, 3.26 ± 0.47 pA, \(n = 6\) vs. NER, 1.73 ± 0.42 pA, \(n = 7\); \(p < 0.05\) in both cases; Fig. 2B) in the presence of NPA.

**Fig. 2 here**

**Similar GAT-1 expression in WIS and NER**

To determine whether the \(I_{\text{tonic}}\) attenuation resulted from altered GAT activity in NER, we compared dentate gyrus GAT expression in WIS and NER (Fig. 3). Despite reduced \(I_{\text{tonic}}\) in the presence of GAT blockers, GAT-1 expression, the major type of GABA transporter in the dentate gyrus, was similar between WIS and NER.

**Fig. 3 here**

**Attenuated \(I_{\text{tonic}}\) response to \(\delta\) subunit agonists in NER**

\(\text{GABA}_A\)R \(\delta\) subunit expression and subtype assembly have been modified, resulting in altered \(I_{\text{tonic}}\) function and pharmacology in various experimental epilepsy models (36, 42, 54, 60). To determine whether the \(\text{GABA}_A\) receptors that mediate \(I_{\text{tonic}}\) are altered in NER, the effects of 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP) and DS-2 on phasic and tonic \(\text{GABA}_A\) currents were tested and compared in WIS and NER (Fig. 4).

Bath application of THIP (1 µM), a \(\text{GABA}_A\) receptor agonist that preferentially activates the \(\delta\) over the \(\gamma_2\) subunit-containing \(\text{GABA}_A\) receptors (2, 5, 8), caused a significant inward shift in \(I_{\text{holding}}\), an effect that was blocked by the \(\text{GABA}_A\) receptor
blocker BIC in both WIS and NER (Fig. 4A). However, THIP induced significantly less
\( I_{\text{holding}} \) shift in NER (6.43 ± 1.63 pA, \( n = 9 \)) than in WIS (14.37 ± 1.87 pA, \( n = 6 \); \( p < 0.01 \)). Consistent with the difference in \( I_{\text{holding}} \) shift, the THIP-induced RMS increase
was less in NER (from 2.16 ± 0.14 pA to 3.33 ± 0.30 pA, \( n = 9 \)) than in WIS (from
2.41 ± 0.20 pA to 4.46 ± 0.35 pA, \( n = 6 \); \( p < 0.01 \)). In accordance with previous
reports (22), THIP failed to induce a detectable change in IPSC, either in WIS or
NER. The main characteristics of IPSC before and after THIP application were
similar both in WIS (frequency, 1.68 ± 0.23 Hz vs. 1.66 ± 0.27 Hz; amplitude, 60.98 ±
4.15 pA vs. 58.67 ± 5.66 pA; weighted decay time constant, 12.67 ± 0.94 ms vs.
13.88 ± 0.72 ms, \( n = 6 \)) and NER (frequency, 1.29 ± 0.29 Hz vs. 1.39 ± 0.28 Hz;
amplitude, 57.35 ± 5.45 pA vs. 54.74 ± 6.33 pA; weighted decay time constant, 13.88
± 0.72 ms vs. 13.99 ± 0.96, \( n = 9 \)) (\( p > 0.6 \) in all cases).

Similar results were observed with another preferential GABA\( \alpha \)R \( \delta \) subunit
modulator, DS-2 (57). DS-2 induced a significantly smaller \( I_{\text{tonic}} \) increase in NER
(13.36 ± 2.43 pA, \( n = 7 \)) than in WIS (22.26 ± 3.10 pA, \( n = 8 \); \( p < 0.05 \); Fig. 4).
Consistent with the difference in \( I_{\text{holding}} \) shift, DS-2 induced less RMS change in NER
(\( \Delta \text{RMS}, 0.90 ± 0.05 \), \( n = 7 \)) than in WIS (\( \Delta \text{RMS}, 1.48 ± 0.15 \) pA, \( n = 8 \); \( p < 0.05 \)).

These results are in agreement with the notion that distinct tonic inhibition is
mediated by \( \delta \) subunit-containing GABA\( \alpha \)R in DGGCs (30, 48) and suggest \( \delta \) subunit
down-regulation in NER.

Fig. 4 here

Reduced effects of allopregnanolone on \( I_{\text{tonic}} \) but not on \( I_{\text{phasic}} \) in NER
Despite reduced $I_{\text{tonic}}$ in NER, IPSC amplitude and decay time kinetics were not significantly different between WIS and NER (Fig. 1), suggesting the selective alteration of the $\delta$ subunit-containing GABA$_A$ receptors over the $\gamma_2$ subunit-containing receptors in NER. At a high concentration of 1 $\mu$M, allopregnanolone (ALLO) potentiates $I_{\text{tonic}}$ and prolongs IPSC decay time (22, 34). To further determine whether the GABA$_A$ receptors mediating $I_{\text{tonic}}$, as opposed to the receptors mediating $I_{\text{phasic}}$, were selectively altered in NER, we tested and compared $I_{\text{tonic}}$ facilitation and prolongation of spontaneous IPSC decay time by administering 1 $\mu$M ALLO in WIS and NER (Fig. 5).

Bath application of ALLO increased $I_{\text{tonic}}$, as shown by a significant inward shift in $I_{\text{holding}}$ and RMS increase both in WIS and NER ($p < 0.01$ in all cases), and the effects were blocked by the GABA$_A$R blocker BIC (Fig. 5A). However, ALLO caused a significantly smaller $I_{\text{holding}}$ shift in NER ($12.52 \pm 4.61$ pA, $n = 7$) than in WIS ($30.45 \pm 6.57$ pA, $n = 6$; $p < 0.05$). The ALLO-induced RMS increase in NER (from $1.89 \pm 0.19$ pA to $4.06 \pm 0.74$ pA, $n = 7$) was less than that in WIS (from $2.41 \pm 0.13$ pA to $5.70 \pm 0.98$ pA, $n = 6$; $p < 0.05$; Fig. 5A and 5B). ALLO also prolonged the IPSC decay time (Fig. 5C and 5D), with no detectable change in the frequency or amplitude of IPSC in either group. In contrast to the lesser $I_{\text{tonic}}$ facilitation in NER, ALLO prolonged the IPSC decay time at a similar rate in WIS ($246.0 \pm 66.1\%$ of control, $n = 6$) and NER ($239.7 \pm 64.0\%$ of control, $n = 7$; $p > 0.9$).

The results suggest that extrasynaptic GABA$_A$ receptors mediating $I_{\text{tonic}}$ are down-regulated, whereas synaptic receptors mediating $I_{\text{phasic}}$ are relatively preserved in the DGGCs of NER.

*Fig. 5 here*
Selective reduction of GABA\(_A\)R \(\delta\) subunit expression over \(\gamma_2\) subunit expression in NER

To confirm the selective down-regulation of extrasynaptic GABA\(_A\) receptors mediating \(I_{\text{tonic}}\) in NER DGGCs, we compared the expression of GABA\(_A\)R \(\delta\) and \(\gamma_2\) subunits in the dentate gyrus of NER with those of WIS. In both WIS and NER, Western blot analysis showed the presence of GABA\(_A\)R \(\delta\) and \(\gamma_2\) subunits in the dentate gyrus (Fig. 6A). However, \(\delta\) subunit polypeptide expression in NER was significantly less than in WIS, whereas \(\gamma_2\) subunit expression was similar between WIS and NER (Fig. 6B).

*Fig. 6 here*
The main findings in the present study are as follows: 1) $I_{\text{tonic}}$ amplitude was attenuated with diminished IPSC frequency in the DGGCs of spontaneous epileptic rats, NER, compared with those of the normal parent strain, WIS; 2) $\delta$-subunit-containing GABA$_A$Rs mediating $I_{\text{tonic}}$ were less functional in NER than in WIS, whereas GABA$_A$Rs mediating $I_{\text{phasic}}$ were relatively preserved; and 3) GABA transporters may not be major factors causing $I_{\text{tonic}}$ reduction in NER. Taken together, these findings suggest that NER is a novel epileptic model showing a combination of decreased presynaptic GABA release, reduced $I_{\text{tonic}}$, and reduced $I_{\text{tonic}}$ sensitivity to neurosteroids in DGGCs, all of which increase seizure vulnerability in the spontaneous epileptic rat.

$I_{\text{tonic}}$ amplitudes vary depending on several conditions, including extracellular GABA concentrations, in slice preparations [see review, (14)]. For example, $I_{\text{tonic}}$ in DGGCs of adult male rats range from $2.3 \pm 0.3$ pA (59) to $72 \pm 0.3$ pA (27) in normal ACSF. $I_{\text{tonic}}$ in DGGCs was relatively small, even in control WIS ($3.36 \pm 0.43$ pA, $n = 13$), in our slice preparations. However, given that extra- and/or peri-synaptic GABA$_A$Rs mediating $I_{\text{tonic}}$ are in a preferred position for activation by extracellular GABA (12), our results showing the consistent decrease of $I_{\text{tonic}}$ in NER support the notion that CSF and brain GABA are reduced in correlation with epileptic sensitivity (26). With several diffusion parameters (53), the amount of GABA molecules that diffuse over some distance and activate the high-affinity GABA$_A$Rs responsible for $I_{\text{tonic}}$ is dependent on the number of vesicles released into the synaptic cleft. GABA terminals are reduced in human and experimental epilepsy (18, 40), although no consistent changes in the epileptic human hippocampus have been demonstrated.
Our results showing a decrease in IPSC frequency are in line with the explanation that GABA terminals are decreased in the DGGCs of NER. However, we cannot exclude the possibility that IPSC frequency was decreased due to presynaptic changes, including decreased spontaneous firing of afferent GABA interneurons without a reduction in GABA terminals in NER. Nevertheless, our finding showing the attenuation of $I_{\text{tonic}}$ amplitudes with decreased IPSC frequency in NER supports the notion that $I_{\text{tonic}}$ is mediated by GABA released from the presynaptic vesicles responsible for activating IPSCs in the hippocampus (15).

In contrast to decreased presynaptic GABA release, our results challenge the notion that increased GABA clearance by GAT activity decreases extracellular GABA concentration, in turn attenuating $I_{\text{tonic}}$ in NER. With the attenuated $I_{\text{tonic}}$ in the presence of membrane GAT blockers (Fig. 2), it is not likely that increased GAT activity reduced $I_{\text{tonic}}$ in NER. This idea was confirmed by the lack of difference in GAT-1 expression in the dentate gyrus between NER and WIS (Fig. 3). (1). Similarly, GAT-1-mediated GABA uptake and clearance are unaffected in both DGGCs and CA1 neurons in the pilocarpin model of epilepsy (13).

Genetic variation in the GABA$_A$R $\delta$ subunit contributes to human general epilepsy via a decrease in GABA$_A$ current (7). Our results showing attenuated $I_{\text{tonic}}$ amplitudes with their reduced response to the $\delta$ subunit-specific agonists THIP and DS-2 (Fig. 4) suggest that GABA$_A$Rs are altered in NER DGGCs. It is noteworthy that agonist-induced $I_{\text{tonic}}$ was attenuated under conditions of reduced GABA release in NER. Different ambient GABA levels influence the pharmacology of GABA$_A$R modulators (19). Indeed, THIP-activated $I_{\text{tonic}}$ is significantly enhanced when the ambient GABA level is reduced, such as when GABA release is decreased by
tetrodotoxin in cerebellar granule cells. Given that the ambient GABA level was reduced in NER, as shown by the decreased sIPSC frequency, attenuated THIP-activated $I_{\text{tonic}}$ suggests that $\delta$ subunit-containing receptors are decreased in NER. This idea was further supported by the decreased $\delta$ subunit expression in the dentate gyrus of NER in the present study.

In chemically induced temporal lobe epilepsy models, a consistent pattern of GABA$_A$R subunit changes includes decreased expression of the $\delta$ subunit along with increased expression of the $\gamma_2$ and $\alpha_4$ subunits in the dentate gyrus (28, 36, 42). Given that the $\gamma_2$ subunit plays a major role in phasic inhibition (30), these changes could alter both tonic and phasic inhibition in the dentate gyrus. However, our results, which showed no differences in the amplitude and decay time kinetics of IPSCs in WIS and NER, suggest that the functions of $\gamma_2$ subunits are relatively preserved in NER. This notion was further supported by results showing similar $\gamma_2$ subunit polypeptide expression in WIS and NER in the present study. Similar GABA$_A$Rs changes were observed in epileptic mutant mouse stargazers, where the $\alpha_1$, $\beta_2$, and $\gamma_2$ subunits mediating $I_{\text{phasic}}$ were essentially unaffected, whereas the $\delta$ subunit expression in the dentate gyrus was reduced (35). Our data showing that 1 µM ALLO induced less facilitation of $I_{\text{tonic}}$ in NER than in WIS but caused similar prolongation of sIPSC decay time in the two groups (Fig. 5) further support the idea that $\delta$ subunit-containing GABA$_A$Rs mediating $I_{\text{tonic}}$ are less functional in NER than in WIS, whereas GABA$_A$Rs mediating $I_{\text{phasic}}$ are relatively preserved. However, we could not exclude the possibility that changes in the $\alpha_5\gamma_2$ receptors that mediate $I_{\text{tonic}}$ in NER led to the small average reduction in the $\gamma_2$ subunit expression in NER (Fig. 6). Future studies are warranted to determine the exact molecular mechanism responsible for the
selective down-regulation of extrasynaptic GABA<sub>A</sub>R function in spontaneous epileptic models.

Although down-regulation of the δ subunit does not always result in decreased I<sub>tonic</sub> in chemically induced epileptic animals (60), it is notable that I<sub>tonic</sub> mediated by δ subunit-containing GABA<sub>A</sub>Rs is a selective target for low concentrations of neurosteroids (4, 47). Indeed, a reduced amount of GABA<sub>A</sub>R δ subunits and, in turn, attenuated neurosteroid modulation in tonic GABA<sub>A</sub> inhibition, increases seizure vulnerability in animals (36). Combined with these results and the knowledge that neurosteroids are increased in the brain under various stress conditions (37), our data showing the I<sub>tonic</sub> decrease by down-regulation of the δ subunit suggest that, in addition to a basal I<sub>tonic</sub> decrease, reduced I<sub>tonic</sub> facilitation in various stress condition renders NER more susceptible to seizure generation. This notion was supported by the results showing that ALLO facilitation of I<sub>tonic</sub> was much less in NER than in WIS in the present study.

In the present study, epileptic responses were estimated by audiogenic stimulation in NER to confirm seizure susceptibility. However, NER are innately hypersensitive to a variety of audiogenic and nonaudiogenic stressors (20, 29). In fact, NER show a greater propensity for generalized seizures via nonaudiogenic stimuli (i.e., pentylenetetrazol, tossing, and transcorneal electroshock). In genetically epilepsy-prone rats (GEPRs), nonaudiogenic seizures are associated with abnormalities in the hippocampus, among other structures (10, 55, 56), whereas audiogenic seizures are limited to the inferior colliculus (6, 11). Thus, GABA<sub>A</sub> inhibition deficits in DGGCs are likely related to nonaudiogenic seizures in NER.

Given that prolonged epileptiform bursting depolarization produces persistent
changes in GABA_A current, audiogenic and spontaneous seizure activity may have caused the selective reduction of GABA_A receptor δ subunit and, in turn, I_{tonic} attenuation in NER. However, this is not likely the case in the present study because the post-synaptic GABA_A R changes induced by prolonged depolarization enhance I_{tonic} (39) or reduce the surface expression of the γ_2 subunit, but not the δ subunit (16). Overall, our data showed deficits in phasic and tonic GABA_A inhibition in DGGCs, which may increase seizure vulnerability in NER. The spontaneously epileptic rat is a useful model of generalized tonic–clonic seizure, which mimics hippocampal GABA abnormalities of human epilepsy.

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DISCLOSURES

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

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Fig. 1. Decreased tonic and phasic GABA_A inhibition in Noda epileptic rats. A: Representative Wistar and Noda epileptic rat (NER) dentate gyrus granule cell (DGGC) current traces indicating the presence of tonic GABA_A currents following the application of 20 μM bicuculline (BIC) in normal aCSF. B: Comparison of the tonic current amplitude in Wistar and Noda epileptic rats in normal aCSF and in the presence of 3 μM GABA, respectively. C: Expanded current traces illustrating spontaneous IPSCs obtained from the same neuron as in A. D, E, and F: Summary data showing sIPSC frequency (D), amplitude (E), and decay time (E) are shown. Summarized data shown are means ± SEM. *p < 0.05, and **p < 0.01 compared with Wistar rats.
Fig. 2. GABA transporter blockers enhance $I_{\text{tonic}}$ in Wistar and Noda epileptic rats. A: Representative Wistar and Noda epileptic rat (NER) dentate gyrus granule cell (DGGC) current traces showing the effect of NO-711 (5 μM), a GABA transporter blocker, on $I_{\text{holding}}$ and RMS. Note that the effects of NO-711, which were blocked by BIC, were smaller in NER than in Wistar rats. B and C: Mean changes in $I_{\text{holding}}$ (B) and RMS (C) induced by BIC in the presence of two different GAT blockers, NO-711 and nipecotic acid (NPA), are summarized. Summarized data shown are mean ± SEM ($n = 6–7$). *$p < 0.05$, and **$p < 0.01$ compared with Wistar rats.
Fig. 3. Comparison of GABA transporter 1 expression in dentate gyrus of Wistar and Noda epileptic rats. A: Western blot analysis showing similar neuronal GABA transporter (GAT-1) expression in the dentate gyrus of Wistar and Noda epileptic rats (NER). B: Summarized GAT-1 expression in WIS and NER. GAT-1 expression was normalized to the level detected in Wistar rats \((n = 5)\) and compared with the expression in NER \((n = 6)\). Note that the expression levels were not different between the two groups.
Fig. 4. Effects of GABA<sub>A</sub>R δ subunit modulators on DGCC I<sub>tonic</sub> in Wistar and Noda epileptic rats. A: Representative current traces from Wistar and Noda epileptic rats (NER) showing that THIP (1 μM) induced an inward shift in I<sub>holding</sub> and increased RMS, which was blocked by the GABA<sub>A</sub> receptor antagonist, bicuculline (BIC). B and C: Mean changes in I<sub>holding</sub> (B) and RMS (C) induced by two different GABA<sub>A</sub>R δ subunit selective drugs, THIP and DS-2, respectively, are summarized. Summarized data shown are means ± SEM (n = 6–8). *p < 0.05 and **p < 0.01 compared with Wistar rats.
Fig. 5. Effects of allopregnanolone on dentate gyrus granule cell (DGGC) $I_{\text{tonic}}$ and $I_{\text{phasic}}$ in Wistar and Noda epileptic rats. A: Representative current traces in Wistar and Noda epileptic rats (NER) showing that allopregnanolone (ALLO, 1 $\mu$M) induced an inward shift in $I_{\text{holding}}$ and increased RMS, which were blocked by the GABA$_A$ receptor antagonist bicuculline (BIC). B: Mean changes in $I_{\text{holding}}$ and RMS induced by ALLO are summarized. C: Averaged IPSCs ($n = 80$ events) obtained from the same neuron in NER as in A before and during bath application of ALLO. D: Mean changes in IPSC decay time constants are summarized in Wistar ($n = 6$) and NER ($n = 7$), respectively. Summarized data shown are means ± SEM. *$p < 0.05$ compared with the respective control.
Fig. 6. GABA<sub>A</sub>R δ subunit expression in the dentate gyrus of Wistar and Noda epileptic rats. A: Representative Western blot analysis showing GABA<sub>A</sub>R δ subunit expression in Wistar (WIS) and Noda epileptic rats (NER). B: GABA<sub>A</sub>R δ and γ<sub>2</sub> subunit expression in WIS and NER was summarized and compared. The protein expression was normalized to the level detected in WIS and compared with the expression in NER. Summarized data shown are means ± SEM (n = 11 and n = 5 for γ<sub>2</sub> and δ subunit, respectively). *p < 0.05 compared with Wistar rats.
Fig. 3 - Sudip et al

A

GAT1 (67kDa) [Image]
Actin (43kDa) [Image]

B

Protein expression [Image]
Fig. 4- Sudip et al

A

Wistar

THIP

BIC

NER

20 pA

1 min

B

I\text{holding change} (pA)

\begin{align*}
\text{Wistar} & \quad \text{NER} \\
\text{THIP} & \quad 10 \quad 10 \quad * \\
\text{DS-2} & \quad 20 \quad 10 \quad **
\end{align*}

C

RMS increase (pA)

\begin{align*}
\text{Wistar} & \quad \text{NER} \\
\text{THIP} & \quad 2 \quad 2 \quad ** \\
\text{DS-2} & \quad 1 \quad 1 \quad *
\end{align*}
**Fig. 5 - Sudip et al**

**A**  
*Wistar*  

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**B**  

![Bar chart with data points for Wistar and NER, showing statistical significance (*) in the holding current change and RMS increase.](chart_b.png)

**C**  

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**D**  

![Graph showing weighted τ (msec) for Wistar and NER groups, with statistical significance (*).](chart_d.png)