Brief Communications

# Which $GABA_A$ Receptor Subunits Are Necessary for Tonic Inhibition in the Hippocampus?

**Joseph Glykys,**[1,2] **Edward O. Mann,**[2] **and Istvan Mody**[1,2]

[1]Interdepartmental PhD Program for Neuroscience and [2]Department of Neurology, David Geffen School of Medicine at the University of California, Los Angeles, California 90095

**$GABA_A$ receptors ($GABA_A$Rs) assembled of different subunits mediate tonic and phasic inhibition in hippocampal neurons. CA1/CA3 pyramidal cells (PCs) predominantly express $\alpha$5 subunits whereas dentate gyrus granule cells (DGGCs) and molecular layer (ML) interneurons predominantly express $\delta$ subunits. Both $\alpha$5- and $\delta$-containing $GABA_A$Rs mediate tonic inhibition. We have shown previously that mice lacking $\alpha$5 subunits (*Gabra5*$^{-/-}$) have a residual tonic current in CA1/CA3 PCs because of an upregulation of $\delta$ subunits, but the basis of the residual tonic current in DGGCs and ML interneurons of mice lacking the $\delta$ subunit (*Gabrd*$^{-/-}$) is still unknown. We now show that wild-type DGGCs have a small tonic current mediated by $\alpha$5 subunit-containing $GABA_A$Rs responsible for ~29% of the total tonic current. To better identify the $GABA_A$Rs mediating tonic inhibition in hippocampal neurons, we generated mice lacking both $\alpha$5 and $\delta$ subunits (*Gabra5/Gabrd*$^{-/-}$). Recordings from CA1/CA3 PCs, DGGCs, and ML interneurons in these mice show an absence of tonic currents without compensatory changes in spontaneous IPSCs (sIPSCs), sEPSCs, and membrane resistance. The absence of tonic inhibition results in spontaneous gamma oscillations recordable *in vitro* in the CA3 pyramidal layer of these mice, which can be mimicked in wild-type mice by blocking $\alpha$5 subunit-containing $GABA_A$Rs with 50 nM L-655,708. In conclusion, depending on the cell type, the $\alpha$5 and $\delta$ subunits are the principal $GABA_A$R subunits responsible for mediating the lion's share of tonic inhibition in hippocampal neurons.**

*Key words:* **tonic inhibition; $\alpha$5; $\delta$; hippocampus; gamma oscillations; phasic inhibition**

## Introduction

Considerable kinetic and pharmacological differences exist between tonic and phasic inhibition mediated by perisynaptically and extrasynaptically located $\alpha$5 and $\delta$ subunit-containing $GABA_A$Rs and by $GABA_A$Rs located at synapses respectively (Mody and Pearce, 2004; Farrant and Nusser, 2005). The $\delta$ subunit is present in the neocortex, thalamus, striatum, dentate gyrus granule cells (DGGCs) and cerebellar granule cells where it mediates tonic inhibition (Nusser et al., 1998; Pirker et al., 2000; Nusser and Mody, 2002; Stell et al., 2003). The $\alpha$5 subunit mediates tonic inhibition in CA1/CA3 pyramidal cells (PCs) (Pirker et al., 2000; Caraiscos et al., 2004; Glykys and Mody, 2006; Serwanski et al., 2006). Previously, the $\alpha$5-$GABA_A$Rs were thought to be the sole mediators of tonic inhibition in hippocampal CA1/CA3 PCs, whereas the tonic inhibition of DGGCs was predominantly mediated by $\delta$-$GABA_A$Rs (Stell et al., 2003; Caraiscos et al., 2004). Yet, in the presence of 5 $\mu$M GABA, mice lacking $\alpha$5 subunits (*Gabra5*$^{-/-}$) showed a residual tonic inhibition in CA1/CA3 PCs mediated by an upregulation of $\delta$ subunits (Glykys and Mody, 2006), and mice lacking $\delta$ subunits (*Gabrd*$^{-/-}$) expressed small residual tonic inhibitory currents in DGGC and molecular layer (ML) interneurons (Stell et al., 2003; Glykys et al., 2007) mediated by a yet unidentified $GABA_A$R subunit. $GABA_A$Rs containing $\varepsilon$ subunits have been proposed to contribute to tonic inhibition in CA3 PCs through openings that do not always require the presence of an agonist (McCartney et al., 2007), and $GABA_A$Rs formed solely of $\alpha$ and $\beta$ subunits have been shown to mediate a small fraction of tonic inhibition in CA1 PCs (Mortensen and Smart, 2006).

To determine the precise fractional contribution of the two principal $GABA_A$R subunits ($\alpha$5 and $\delta$) to the tonic inhibition in hippocampal neurons, we generated $\alpha$5 and $\delta$ $GABA_A$R subunit double knock-out mice (*Gabra5/Gabrd*$^{-/-}$). We demonstrate that $\alpha$5 and $\delta$ are the main subunits responsible for tonic inhibition in CA1/CA3 PCs, DGGCs, and ML interneurons. Furthermore, a complete abolishment of tonic inhibition in CA3 PCs results in spontaneous gamma oscillations recordable *in vitro*.

## Materials and Methods

*Slice preparation.* Adult (>35-d-old) male C57BL/6, *Gabrd*$^{-/-}$ (Jackson Laboratories, Bar Harbor, ME) and *Gabra5*$^{-/-}$ (original breeding pairs generously supplied by Merck, Sharp and Dohme, Harlow, UK) were used. *Gabra5/Gabrd*$^{-/-}$ double knock-out mice on C57BL/6 background were generated by cross-breeding *Gabra5*$^{-/-}$ and *Gabrd*$^{-/-}$ mice, bred and raised under the care of the University of California, Los Angeles (UCLA), Division of Laboratory Animal Medicine. PCR was performed on DNA isolated from tail samples of the different genotypes. PCR of *Gabra5/Gabrd*$^{-/-}$ samples were run twice in the presence of specific $\alpha$5 and $\delta$ primers. Mice were anesthetized with halothane and decapitated according to a protocol approved by the UCLA Chancellor's

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Correspondence should be addressed to Istvan Mody at the above address. E-mail: mody@ucla.edu.

J. Glykys's present address: Department of Neurology, Massachusetts General Hospital, CNY B-114, Charleston, MA 02129.

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Animal Research Committee. The brain was removed and placed in ice-cold artificial CSF (aCSF; used for cutting, incubating and recording) containing (in mM) 126 NaCl, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 1.25 $NaH_2PO_4$, 26 $NaHCO_3$, and 10 D-glucose, with pH 7.3–7.4 when bubbled with 95% $O_2$ and 5% $CO_2$. Coronal and horizontal brain slices, 350-$\mu$m-thick, were cut with a Leica (Wetzlar, Germany) VT1000S Vibratome, in aCSF containing 3 mM kynurenic acid (Sigma, St. Louis, MO). Slices were placed into an interface holding chamber at 30 ± 1°C, and for some DGGC recordings, slices were kept in a submerged chamber.

*Electrophysiology.* For whole-cell recordings, slices were transferred to a submerged recording chamber, and perfused (~4–5 ml/min) at 32–34°C with 95% $O_2$ and 5% $CO_2$ saturated aCSF. CA1/CA3 PCs, DGGC and ML interneurons were visually identified by IR-DIC videomicroscopy (Olympus, Melville, NY; BX51WI, 40× water-immersion objective) and recorded with an Axopatch 200A amplifier (Molecular Devices, Sunnyvale, CA). For measurements of tonic inhibition, kynurenic acid (3 mM) and GABA (5 $\mu$M) were included in the aCSF. Microelectrodes (3–5 MΩ) contained the following internal solution (in mM): 140 CsCl, 1 $MgCl_2$, 10 HEPES, 0.1 EGTA, 4 NaCl, 2 MgATP, 0.3 NaGTP, and 5 QX-314 (only for CA1/CA3 PCs), pH ~7.27, ~275 mOsm. Spontaneous EPSCs (sEPSCs)/sIPSCs were recorded with the following (in mM): 140 cesium-methylsulfonate, 10 HEPES, 0.2 EGTA, 5 NaCl, 2 MgATP, and 0.2 NaGTP, ~271 mOsm, pH ~7.29. Voltage ramps were measured with the following (in mM): 135 K-gluconate, 5 KCl, 10 HEPES, 2 $MgCl_2$, 0.1 EGTA, 4 ATP-Na, and 0.3 GTP-Na. Series resistance and whole-cell capacitance were estimated and compensated to 70–80% (lag 7 $\mu$s). Recordings were discontinued if series resistance increased by >25%. Network activity was recorded in an interface chamber at 33–35°C using patch pipettes filled with aCSF connected to a differential amplifier (Model 3000; A-M Systems, Carlsborg, WA) with a high-pass filter at 0.1 Hz.

*Data acquisition and analysis.* All recordings were low-pass filtered at 3 kHz and digitized on-line at 10 Hz using a PCI-MIO 16E-4 data acquisition board (National Instruments, Austin, TX). Tonic current measurement was determined as described previously (Glykys and Mody, 2007). (1) The baseline mean current ($I_{mean}$) was obtained by fitting a Gaussian to the all-points histogram over 1 s epochs (see Glykys and Mody, 2007). (2) Input resistance in CsCl-filled cells was calculated during the currents produced by seal tests. When K-gluconate was used, input resistance was estimated by the slope of a line fitted to the I/V plot obtained from a ramp protocol (−46 to −174 mV, 1 s). (3) sPSCs were detected in 30–60 s recording segments. Event frequency, 10–90% rise time ($RT_{10-90}$), peak amplitude, and tau weighted ($\tau_w$) values were measured. Detection and analysis were performed using a custom-written LabView-based software (EVAN). (4) For field potential analysis, the power spectral density was calculated for 3 min periods during control conditions and 10 min after drug application. Components caused by line noise were removed with a seven-point median filter, and the resulting power spectrum was used to determine the peak frequency and power area ($V^2$) in the gamma-frequency band (30–80 Hz). The ratio between power in gamma- and ripple-frequency (150–250 Hz) bands was also determined ($\gamma$/rp). All data are shown as mean ± SEM. Statistical analysis was performed by Student's *t* test and one- and two-way ANOVA with Tukey honest significant difference or Dunnett's *post hoc* tests. Significance was set to $p < 0.05$.

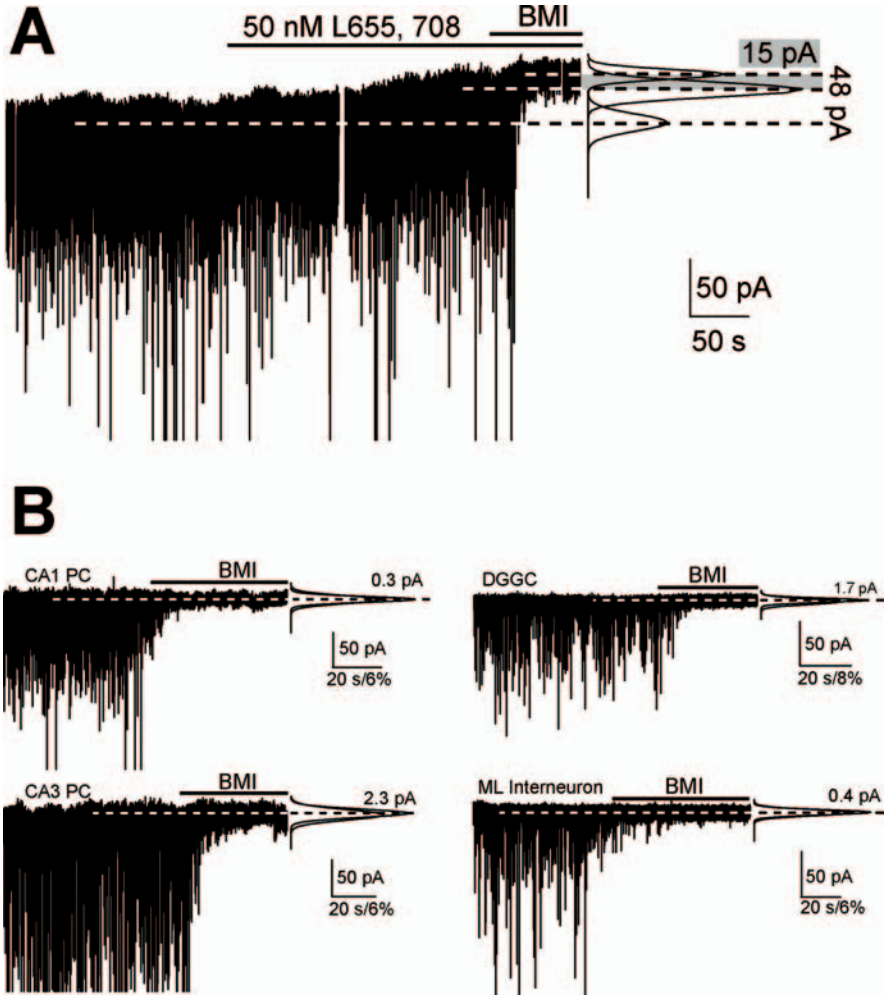


**Figure 1.** $GABA_A$Rs containing $\alpha$5 or $\delta$ subunits mediate tonic inhibitory currents in hippocampal neurons. ***A***, Whole-cell voltage-clamp recording of a CA1 PC ($V_h$ = −70 mV). Top lines indicate the perfusion of L-655,708 and BMI. Right, Fit to the all-points histogram from segments of each of the three conditions. ***B***, Whole-cell voltage-clamp recordings from *Gabra 5/Gabrd*$^{-/-}$ CA1 and CA3 PC, DGGC and a molecular layer interneuron ($V_h$ = −70 mV). Right, Same as in ***A***.

## Results

### Tonic inhibitory current from CA1 PCs is partially mediated by non-$\alpha$5-$GABA_A$Rs

CA1 PCs express $\alpha$5-$GABA_A$Rs mainly at extrasynaptic sites, with low levels of $\delta$ subunit expression (Pirker et al., 2000). CA1 PC tonic inhibitory currents are not significantly altered by compounds known to potentiate $\delta$-$GABA_A$Rs (Stell et al., 2003; Wei et al., 2004), but the cellular components necessary to express functional $\delta$-$GABA_A$R are present (Glykys and Mody, 2006). To assess the component mediated by $\delta$-$GABA_A$Rs in these cells, we measured tonic inhibition after application of L-655,708, an $\alpha$5-$GABA_A$R-selective and high-affinity (Kd ~2.5 nM) imidazo[1,5-$\alpha$]benzodiazepine with antagonist (inverse benzodiazepine agonist) properties (Quirk et al., 1996). L-655,708 (50 nM) reduced the tonic current by 73% ($p = 0.003$, paired *t* test) (Fig. 1*A*, Table 2), and 200 nM had no additional effect (67% reduction, $p = 0.004$ paired *t* test; the residual currents were similar, $p = 0.56$ unpaired *t* test) (Table 2). It appears that $\alpha$5-$GABA_A$Rs contribute little to phasic currents as L-655,708 (50 nM) did not affect sIPSCs (CON, 16.4 ± 1.5 $s^{-1}$, peak 36.8 ± 2.5 pA, $RT_{10-90}$ 0.57 ± 0.02 ms, $\tau_w$ 5.59 ± 0.11 ms; L-655,708, 17.7 ± 1.8 $s^{-1}$,

**Table 1. Phasic inhibitory currents recorded in different neurons from WT and *Gabra5/Gabrd*$^{-/-}$**

| | WT | | | | | | *Gabra5/Gabrd*$^{-/-}$ | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CA1 PC | | CA3 PC | | DGGC | | CA1 PC | | CA3 PC | | DGGC | |
| | sIPSCs | sEPSCs | sIPSCs | sEPSCs | sIPSCs | sEPSCs | sIPSCs | sEPSCs | sIPSCs | sEPSCs | sIPSCs | sEPSCs |
| $I_{mean}$ (pA/sec) | 2.25 ± 0.36 | 0.37 ± 0.04 | 11.8 ± 2.0 | 1.86 ± 0.59 | 1.96 ± 0.72 | 0.32 ± 0.03 | 2.04 ± 0.47 | 0.42 ± 0.05 | 17.2 ± 2.98 | 2.19 ± 0.40 | 1.17 ± 0.41 | 0.32 ± 0.08 |
| Frequency (Hz) | 20.8 ± 3.04 | 3.13 ± 0.71 | 39.0 ± 3.12 | 15.1 ± 0.82 | 12.8 ± 3.67 | 4.84 ± 1.10 | 20.9 ± 3.06 | 4.35 ± 0.64 | 40.0 ± 2.17 | 19.0 ± 2.76 | 10.6 ± 2.45 | 2.81 ± 0.58 |
| Peak amplitude (pA) | 15.8 ± 0.40 | 14.9 ± 1.04 | 32.6 ± 3.04 | 19.5 ± 3.10 | 18.4 ± 1.86 | 12.2 ± 0.28 | 16.2 ± 0.87 | 14.7 ± 1.59 | 41.9 ± 5.59 | 20.1 ± 1.55 | 16.5 ± 1.38 | 11.6 ± 0.94 |
| $RT_{10-90}$ (ms) | 0.78 ± 0.03 | 0.81 ± 0.03 | 1.72 ± 0.09 | 1.15 ± 0.06 | 1.17 ± 0.07 | 0.88 ± 0.05 | 0.69 ± 0.03 | 0.67 ± 0.07 | 1.76 ± 0.08 | 1.22 ± 0.08 | 1.21 ± 0.08 | 0.95 ± 0.03 |
| *Tw* (ms) | 8.45 ± 0.14 | 5.53 ± 0.36 | 9.82 ± 0.39 | 6.27 ± 0.23 | 8.05 ± 0.29 | 4.92 ± 0.14 | 8.22 ± 19 | 5.62 ± 0.17 | 10.7 ± 0.38 | 6.65 ± 0.19 | 8.2 ± 0.34 | 4.78 ± 0.24 |
| *n* | 5 | | 5 | | 8 | | 8 | | 7 | | 7 | |

$I_{mean}$, sIPSCs, and sEPSCs recorded in aCSF with a low $Cl^-$ pipette solution.

**Table 2. Tonic inhibitory current recorded in the different neurons and genotypes**

| Tonic current (pA) | CA1 PC | *n* | CA3 PC | *n* | DGGC | *n* | ML interneurons | *n* |
|---|---|---|---|---|---|---|---|---|
| WT | 38.9 ± 5.10 | 12 | 30.1 ± 5.28 | 8 | 22.0 ± 5.33 | 5 | 12.2 ± 1.68 | 17 |
| *Gabra5*$^{-/-}$ | 23.1 ± 1.75 | 17 | 16.1 ± 2.89 | 4 | 20.3 ± 4.03 | 5 | 13.0 ± 2.79 | 5 |
| *Gabrd*$^{-/-}$ | 36.2 ± 2.17 | 5 | 30.9 ± 4.40 | 5 | 6.59 ± 1.42 | 6 | 12.0 ± 3.46 | 7 |
| *Gabra5/Gabrd*$^{-/-}$ | 1.47 ± 0.59 | 6 | 3.12 ± 1.74 | 4 | 1.56 ± 0.21 | 5 | 1.83 ± 1.75 | 5 |
| WT + 50 nM L-655,708 | 11.3 ± 3.17 | 6 | nd | nd | 15.8 ± 4.1 | 5 | nd | nd |
| WT + 200 nM L-655,708 | 12.6 ± 3.56 | 6 | nd | nd | nd | nd | nd | nd |
| *Gabrd*$^{-/-}$ + 50 nM L-655,708 | 13.2 ± 1.37 | 5 | nd | nd | 1.84 ± 0.69 | 8 | nd | nd |

$I_{mean}$, sIPSCs, and sEPSCs recorded in aCSF with a low $Cl^-$ pipette solution.nd, Not determined

**Table 3. Input resistance recorded in the different neurons and genotypes**

| | CA1 PC | | | CA3 PC | | | DGGC | | | ML interneurons | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Rs* (MΩ) | Control | BMI | *n* | Control | BMI | *n* | Control | BMI | *n* | Control | BMI | *n* |
| WT | 95 ± 8 | 108 ± 8 | 11 | nd | nd | nd | 215 ± 25 | 240 ± 17 | 5 | 182 ± 17 | 200 ± 36 | 7 |
| *Gabra5*$^{-/-}$ | 113 ± 7 | 118 ± 13 | 14 | nd | nd | nd | 217 ± 20 | 290 ± 31 | 5 | 277 ± 45 | 276 ± 52 | 5 |
| *Gabrd*$^{-/-}$ | 95 ± 25 | 121 ± 42 | 6 | 102 ± 22 | 108 ± 28 | 5 | 179 ± 18 | 183 ± 15 | 6 | 192 ± 22 | 180 ± 22 | 4 |
| *Gabra5/Gabrd*$^{-/-}$ | 110 ± 13 | 119 ± 6 | 6 | 87 ± 4 | 91 ± 5 | 4 | 283 ± 24 | 328 ± 27 | 5 | 254 ± 54 | 232 ± 31 | 5 |

No statistical differences observed between WT and the different genotypes in CA1, CA3, DGGC, and ML interneurons in control conditions and in the presence of bicuculline (Dunnett's *post hoc* test, WT used as the control group, $p > 0.05$).

peak 35.3 ± 3.0 pA, $RT_{10-90}$ 0.58 ± 0.03 ms, $\tau_w$ 5.72 ± 0.11 ms; $p > 0.05$; paired *t* test, $n = 6$) or $I_{mean}$ (1 s epochs) (50 nM $p = 0.52$, 200 nM $p = 0.82$, paired *t* test) (Table 1). Thus, under our recording conditions, ~30% of the tonic conductance in wild-type (WT) CA1 PCs is mediated by non-α5-$GABA_A$Rs, most likely δ-$GABA_A$Rs, although receptors composed of only α and β subunits cannot be excluded (Mortensen and Smart, 2006).

In *Gabrd*$^{-/-}$ mice, perfusion of 50 nM L-655,708 reduced CA1 PC, tonic inhibition by 63% ($n = 5$; $p < 0.001$) (Table 2) suggesting that in these mice, some of the tonic inhibitory current is mediated by non-α5/non-δ-containing $GABA_A$Rs, perhaps by receptors composed of only α and β subunits (Mortensen and Smart, 2006), or alternatively, by some α5-$GABA_A$Rs insensitive to L-655,708. To address this, we cross-bred *Gabra5*$^{-/-}$ and *Gabrd*$^{-/-}$ mice to obtain animals lacking both α5- and δ-$GABA_A$Rs (*Gabra5/Gabrd*$^{-/-}$).

### Hippocampal neurons from *Gabra5/Gabrd*$^{-/-}$ mice lack tonic inhibitory currents

CA1 PCs from *Gabra5/Gabrd*$^{-/-}$ mice in 3 mM kynurenic acid and 5 μM GABA showed a complete absence of tonic inhibitory conductance compared with WT mice ($p < 0.0001$) (Fig. 1*B*, 2, Table 2). The input resistance of *Gabra5/Gabrd*$^{-/-}$ CA1 PCs did not change after adding bicuculline (Table 3). Input resistance was also similar between WT and *Gabra5/Gabrd*$^{-/-}$ CA1 PCs when recorded in the presence of 0.5 μM TTX, 50 μM $CdCl_2$, 3 mM kynurenic acid and 10 μM gabazine with K-gluconate internal solution (113 ± 10 MΩ, $n = 8$ vs 132 ± 16 MΩ, $n = 7$, respectively; $p = 0.33$, unpaired *t* test). The lack of tonic conductance in the absence of α5 and δ subunits is in sharp contrast to the condition when only one of the subunits is deleted (Fig. 2). With such a dramatic reduction of tonic inhibition, we wanted to determine whether compensatory changes might occur in sEPSCs or sIPSCs. Event frequencies and kinetics recorded from CA1 PCs in regular aCSF and low chloride internal solution were no different between WT and *Gabra5/Gabrd*$^{-/-}$ CA1 PCs (Table 1). Excitatory and inhibitory $I_{mean}$ (1 s epochs) were similar between WT and *Gabra5/Gabrd*$^{-/-}$ ($p = 0.74$; unpaired *t* test) (Table 1).

Tonic inhibitory currents were lacking in CA3 PCs of *Gabra5/Gabrd*$^{-/-}$ mice ($p = 0.0001$; unpaired *t* test) (Fig. 1*A*, 2, Table 2). Also, *Gabra5/Gabrd*$^{-/-}$ CA3 PCs input resistance did not change after addition of bicuculline (Table 3), and synaptic events did not show differences between the genotypes (Table 1). Thus, deletion of both α5 and δ subunits in CA1 and CA3 PCs produces a complete lack of tonic inhibitory current and no compensatory changes in phasic conductances. Apparently, neither subunit makes a functional contribution to currents generated at GABAergic synapses.

### The α5 subunit also participates in tonic inhibitory currents in both DGGCs and ML interneurons

A small residual tonic current remains in DGGCs of mice lacking δ subunits (Stell et al., 2003; Wei et al., 2004). This small residual current was reduced to noise levels after application of L-655,708 in *Gabrd*$^{-/-}$ DGGCs (Table 2) ($p = 0.02$, unpaired *t* test) sug-

gesting that most, if not all, of this residual tonic current is mediated by $\alpha$5-GABA$_A$Rs. Tonic inhibition was also decreased when 50 nM L-655,708 was applied onto WT DGGCs ($p = 0.01$, paired $t$ test) (Table 2) strongly suggesting that $\alpha$5-GABA$_A$Rs mediate a portion (~29%) of the tonic current recorded in DGGCs, but this fraction could be an underestimate if some $\alpha$5-GABA$_A$Rs are insensitive to L-655,708. We turned to the *Gabra5/Gabrd*$^{-/-}$ mice to address this issue. Tonic inhibitory current was lacking in DGGCs of *Gabra5/Gabrd*$^{-/-}$ mice ($p = 0.003$, unpaired $t$ test) (Fig. 1*B*, 2, Table 2). Input resistance of *Gabra5/Gabrd*$^{-/-}$ DGGCs was similar before and after bicuculline application (Table 3) ($p = 0.83$, paired $t$ test). These results indicate that WT DGGCs express functional GABA$_A$Rs containing the $\alpha$5 subunit which contribute ~29% of the total tonic inhibition. Moreover, assemblies containing the $\alpha$5- and/or $\delta$-GABA$_A$Rs are the only ones mediating tonic current in DGGCs. sIPSCs and sEPSCs properties and $I_{mean}$ were not different between DGGCs of WT and *Gabra5/Gabrd*$^{-/-}$ mice (Tables 1–3).

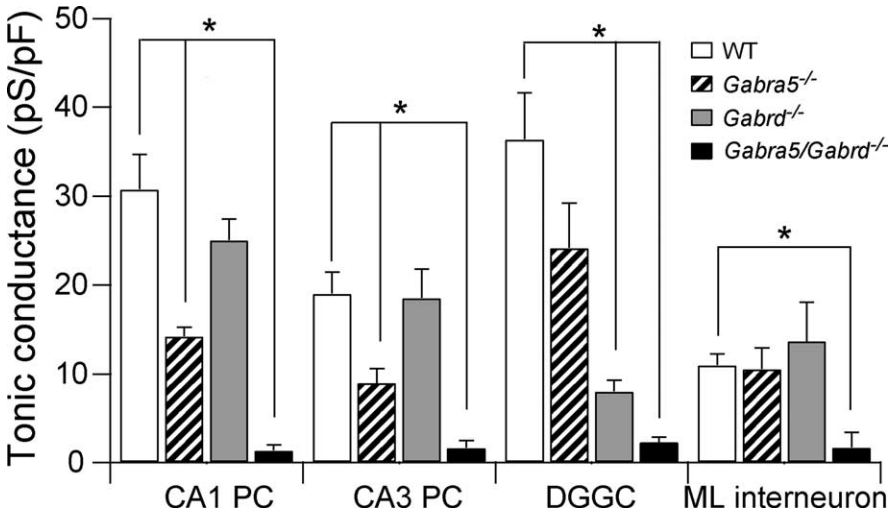


**Figure 2.** Hippocampal neurons from *Gabra5/Gabrd*$^{-/-}$ mice show no tonic inhibitory currents. Mean tonic inhibitory conductance recorded from CA1/CA3 PCs, DGGCs and ML interneurons. One-way ANOVA of each cell type indicate statistical significance $p < 0.05$. Asterisk indicates Dunnett's *post hoc* test being statistically significant ($p < 0.05$) using WT as the control group. The number of cells recorded is indicated in Tables 1–3.

Finally, the ML layer interneurons with a high expression of $\delta$ subunits also show a residual tonic current in *Gabrd*$^{-/-}$ mice (Glykys et al., 2007). Akin to the three other hippocampal cell types recorded in *Gabra5/Gabrd*$^{-/-}$ mice, ML interneurons lacked tonic inhibitory current ($p = 0.002$ unpaired $t$ test) (Fig. 1*B*, 2, Table 1). Perfusing bicuculline did not change the input resistances of ML interneurons in *Gabra5/Gabrd*$^{-/-}$ mice (Table 2) ($p = 0.56$, paired $t$ test).

Together, these results indicate that $\alpha$5- and $\delta$-GABA$_A$Rs mediate tonic inhibition in hippocampal neurons, and at least in adult animals, there is a complete lack of tonic inhibitory currents in their absence.

### The absence of tonic inhibition leads to persistent gamma-frequency oscillations in hippocampal CA3

We reported previously that mice lacking $\alpha$5 subunits, but having a residual tonic inhibition, show signs of neuronal hyperexcitability in the CA3 region (Glykys and Mody, 2006). We wanted to determine the excitability in this region in *Gabra5/Gabrd*$^{-/-}$ mice where tonic inhibitory currents are entirely absent.

In the presence of regular aCSF, extracellular field recordings from the CA3 pyramidal layer of hippocampal slices from *Gabra5/Gabrd*$^{-/-}$ mice showed spontaneous gamma oscillations (peak frequency, 43.7 ± 2.39 Hz; power in the $\gamma$-frequency band, 320 ± 93 $\mu V^2$; $\gamma$/rp ratio, 7.65 ± 1.12; $n = 7$) (Fig. 3), although there was no evidence of spontaneous activity in field recordings from the CA1 pyramidal layer ($n = 6$). No spontaneous oscillations were observed in the CA3 of WT mice (power in the $\gamma$-frequency band, 86 ± 36 $\mu V^2$; $\gamma$/rp ratio, 1.95 ± 0.36; $n = 7$), but after perfusion of 50 and 200 nM L-655,708, gamma oscillations emerged in extracellular recordings from the CA3 pyramidal layer (50 nM, 38.9 ± 4.14 Hz, 362 ± 151 $\mu V^2$; $\gamma$/rp ratio, 5.34 ± 0.80; 200 nM, 39.8 ± 3.56 Hz, 715 ± 297 $\mu V^2$; $\gamma$/rp ratio, 4.97 ± 0.70; $n = 7$) (Fig. 3). To confirm that the effects of L-655,708 on spontaneous activity in WT mice were caused specifically by antagonism of $\alpha$5-GABA$_A$R, 50 and 200 nM L-655,708 were also applied to four of seven slices from *Gabra5/Gabrd*$^{-/-}$ mice. To extract changes in the field potential that were specific to $\gamma$-frequencies, statistical analysis was performed on the ratio of the power area within the $\gamma$- and ripple-frequency bands. A two-way repeated measures ANOVA on changes in $\gamma$/rp ratio, with genotype as the factor and drug concentration as the repeated measure, showed a significant effect of genotype ($F_{(1,9)} = 11.74$; $p = 0.008$) and a significant interaction between the effects of L-655,708 and genotype ($F_{(2,18)} = 6.41$; $p = 0.008$), whereas the effect of L-655,708 was itself of only borderline significance ($F_{(2,18)} = 3.54$; $p = 0.05$). *Post hoc* analysis of the differences between the genotypes at each concentration of L-655,708, using multiple-comparison $t$ tests with Bonferroni correction, showed that the significant interaction observed could be explained by a significantly greater $\gamma$/rp ratio in *Gabra5/Gabrd*$^{-/-}$ versus WT mice in control conditions ($t = 4.81$; $p = 0.032$), which was not apparent after application of either 50 nM ($t = 1.37$; $p = 0.641$) or 200 nM L-655,708 ($t = 1.79$; $p = 0.380$). These results indicate that when tonic inhibitory currents are not present in CA3 PCs, spontaneous gamma oscillations are present. This can be induced in WT mice by blocking the $\alpha$5-GABA$_A$Rs with nanomolar concentration of its specific antagonist.

## Discussion

The main conclusions of our study are that (1) the GABA$_A$Rs responsible for tonic inhibition in principal cells of the hippocampus as well as in ML interneurons contain the $\alpha$5 and/or $\delta$ subunits. (2) Functionally, these two GABA$_A$R subunits do not participate in phasic inhibition, and their absence does not result in compensatory alterations in phasic excitation/inhibition, or changes in input resistance. (3) A complete lack of GABAergic tonic conductance in CA3 PCs produces gamma oscillations recordable *in vitro*.

Tonic inhibition is responsible for generating ~75% of the total inhibitory charge received by hippocampal neurons (Mody and Pearce, 2004). This form of inhibition is mediated by GABA$_A$Rs localized outside the synapses with kinetics and pharmacological properties that distinguish them from synaptic receptors (Farrant and Nusser, 2005). The $\delta$-GABA$_A$Rs are localized perisynaptically and extrasynaptically in the hippocampus (Wei et al., 2003) and are thought not to comprise $\gamma$ subunit as the two subunits are mutually

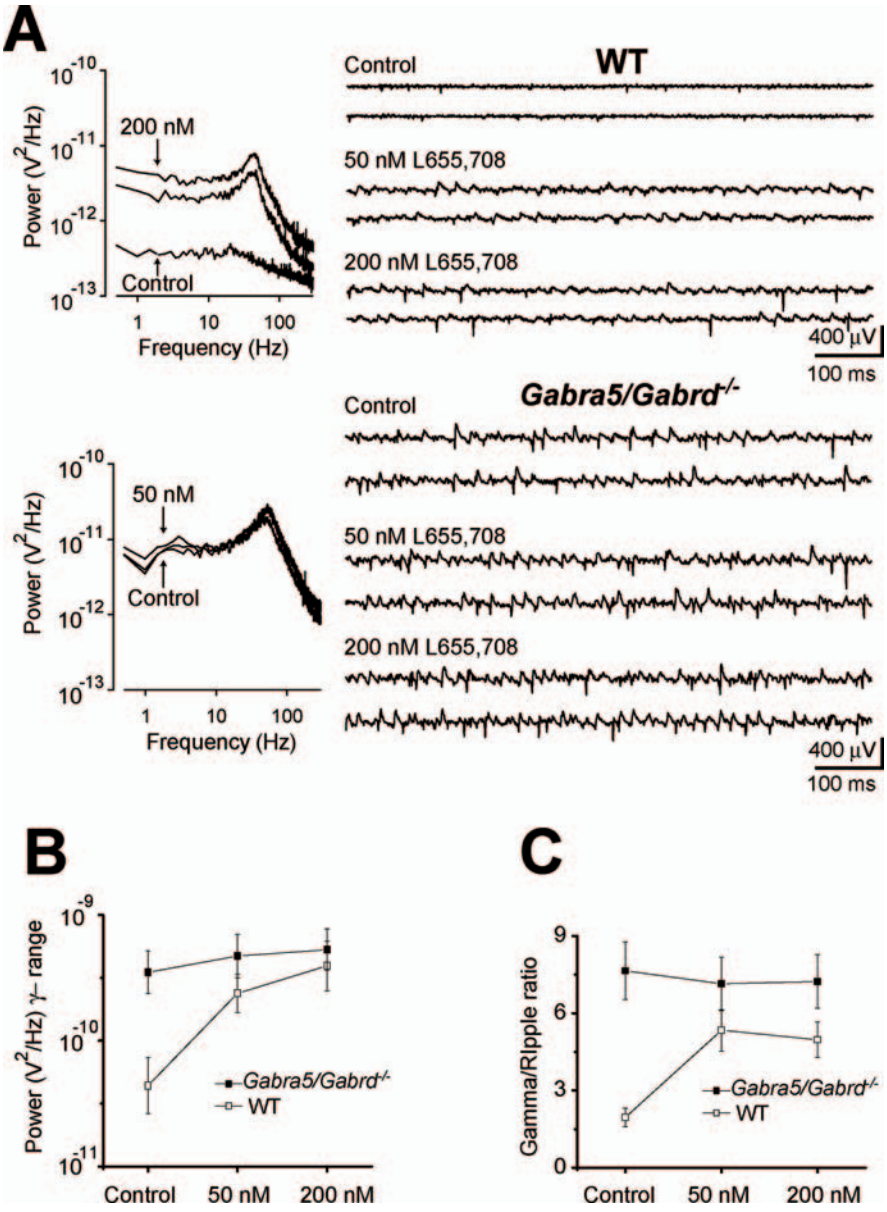


**Figure 3.** Reducing tonic inhibition leads to the emergence of gamma oscillations in hippocampal CA3. Field potential recordings were obtained from the CA3 pyramidal layer of slices maintained in an interface chamber. ***A***, The power spectral density of the field oscillation in control conditions and after cumulative application of 50 and 200 nM L-655,708 in WT (top) and *Gabra5/Gabrd*$^{-/-}$ (bottom) mice. Right, Sample traces notch-filtered at 60 Hz. ***B***, Mean changes in log power in the gamma frequency range (30–80 Hz). ***C***, Mean ratio of the power recorded in the gamma and ripple (150–250 Hz) frequency ranges (WT, $n = 7$; *Gabra5/Gabrd*$^{-/-}$, $n = 4$).

exclusive. The $\alpha$5-GABA$_A$Rs have a more restricted distribution in the brain, and the majority of these receptors mediate tonic inhibition particularly in hippocampal PCs (Glykys and Mody, 2006; Serwanski et al., 2006). When tonic inhibition is absent without compensatory changes in other GABA$_A$Rs, as found in cerebellar granule cells of $\alpha$6 knock-out mice, a conductance of equivalent magnitude is generated by TASK K$^+$ channels (Brickley et al., 2001). No other conductance compensated for the missing tonically active GABA$_A$Rs in *Gabra5/Gabrd*$^{-/-}$ hippocampal neurons because their input resistances in bicuculline were similar to those of WT neurons.

As described previously, DGGCs as well as ML interneurons have a small residual tonic inhibitory current in $\delta$ knock-out mice (Stell et al., 2003; Glykys et al., 2007). Also, CA1 and CA3 PCs show a residual tonic current in the mice lacking $\alpha$5 subunits (Glykys and Mody, 2006), suggesting that other subunits could mediate tonic inhibition in these neurons. The benzodiazepine inverse agonist L-655,708 selectively blocks $\alpha$5-GABA$_A$Rs at nanomolar concentrations (Quirk et al., 1996), and it has been used previously in high concentrations to block currents mediated through $\alpha$5-GABA$_A$Rs (Caraiscos et al., 2004; Scimemi et al., 2005). To avoid possible nonspecific effects on other non-$\alpha$5-GABA$_A$Rs we used concentrations as low as 50 nM to block $\alpha$5 containing receptors and demonstrated that in WT CA1 PCs there is a small tonic inhibitory current mediated by receptors other that $\alpha$5-GABA$_A$Rs, as increasing the dose of L-655,708 (200 nM) did not have any further effects on the tonic current in CA1 PCs. This residual current may be mediated by $\delta$-GABA$_A$Rs, for which there is unfortunately no specific blocker, or by GABA$_A$Rs containing only $\alpha$ and $\beta$ subunits, as it has been shown in cultured rat hippocampal neurons (Mortensen and Smart, 2006). Instead of using various and complex pharmacological approaches, by recording from $\alpha$5/$\delta$ double knock-out mice we show a complete lack of GABAergic tonic inhibition in CA1 and CA3 PCs indicating the need for no other types of GABA$_A$Rs in mediating tonic inhibition.

Although $\delta$-GABA$_A$Rs mediate a large fraction (>70%) of the tonic current in DGGCs, a residual L-655,708-sensitive current can be recorded in $\delta$ knock-out mice. The same amount of tonic current was blocked in WT DGGCs using L-655,708. Previously, the tonic currents recorded in DGGCs have been shown to be potentiated by diazepam, suggesting a participation of $\alpha$5 subunits (Zhang et al., 2007), but probably not any other $\alpha$ subunits, as zolpidem does not potentiate DGGCs tonic current (Nusser and Mody, 2002). The participation of the $\alpha$5-GABA$_A$Rs in the tonic currents of WT DGGCs and the upregulation of these subunits in *Gabrd*$^{-/-}$ ML interneurons was clearly shown in recordings from *Gabra5/Gabrd*$^{-/-}$ mice, where both types of neuron lacked tonic currents.

The $\alpha$5 subunit has been shown previously to be expressed at synaptic sites, albeit at lower levels than found extrasynaptically (Serwanski et al., 2006). Interestingly, we failed to detect differences in fast sIPSCs of *Gabra5*$^{-/-}$ mice (Glykys and Mody, 2006) or in mice with both $\alpha$5 and $\delta$ subunits deleted. In addition, blocking $\alpha$5-GABA$_A$Rs with L-655,708 did not alter phasic inhibition. As previously postulated (Glykys and Mody, 2006), our present findings are consistent with the idea that $\alpha$5-GABA$_A$Rs localized at the synapses are either not functional, or they exist in combinations with other $\alpha$ subunits that confer a different pharmacology to the receptor.

Tonic inhibition has been postulated to have sizeable effects

on the firing and excitability of individual neurons (Farrant and Nusser, 2005), but its effect on network activity has remained obscure. Here we show that the CA3 region of the hippocampus from *Gabra5/Gabrd*$^{-/-}$ mice displays spontaneous gamma oscillations *in vitro*, and that gamma oscillations can also be induced in slices from WT mice by low concentrations of L-605,788. It is known that tonic excitation of the hippocampal CA3, produced by agonists for muscarinic acetylcholine, kainate or metabotropic glutamate receptors, leads to the generation of persistent gamma oscillations in WT mice (Traub et al., 2004), and these results suggest that decreasing tonic inhibition can have similar effects on network activity. The fact that the reductions in tonic inhibition, produced by L-655,708 and the double knock-out of the $\alpha$5 and $\delta$ subunits, were not associated with changes in phasic inhibition, is excellent evidence for the dampening role of tonic inhibition on network excitability. At this point we do not know much about the magnitude and pharmacological properties of tonic inhibition in various CA3 interneurons that are known to be recruited during these oscillations (Mann and Paulsen, 2007). Nevertheless, based on the distribution of $\alpha$5 subunits, the interneurons are unlikely to possess such receptors (Pirker et al., 2000). Therefore, reducing the tonic inhibition of CA3 pyramidal cells alone, as with L-655,708 in WT slices, appears to be sufficient for generating the gamma frequency network oscillations. The prominent oscillations seen in the double knock-outs compared with the hyperexcitability without oscillations observed in *Gabra5*$^{-/-}$ mice (Glykys and Mody, 2006) indicates that a small amount of residual tonic inhibition is capable of stopping the conversion of network hyperexcitability into full-blown synchronous oscillations. Additional research is under way in the *Gabra5/Gabrd*$^{-/-}$ mice to address possible behavioral changes as well as the participation of these two $GABA_A$R subunits in mediating tonic inhibition in other brain areas.

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