Tyrosine Phosphorylation of GABA_A Receptor γ 2-Subunit Regulates Tonic and Phasic Inhibition in the Thalamus

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GABA-mediated tonic and phasic inhibition of thalamic relay neurons of the dorsal lateral geniculate nucleus (dLGN) was studied after ablating tyrosine (Y) phosphorylation of receptor γ 2-subunits. As phosphorylation of γ 2 Y365 and Y367 reduces receptor internalization, to understand their importance for inhibition we created a knock-in mouse in which these residues are replaced by phenylalanines. On comparing wild-type (WT) and γ 2^{Y365/367F} +/– (HT) animals (homozygotes are not viable *in utero*), the expression levels of GABA_A receptor α 4-subunits were increased in the thalamus of female, but not male mice. Raised δ -subunit expression levels were also observed in female γ 2^{Y365/367F} +/– thalamus. Electrophysiological analyses revealed no difference in the level of inhibition in male WT and HT dLGN, while both the spontaneous inhibitory postsynaptic activity and the tonic current were significantly augmented in female HT relay cells. The sensitivity of tonic currents to the δ -subunit superagonist THIP, and the blocker Zn²⁺, were higher in female HT relay cells. This is consistent with upregulation of extrasynaptic GABA_A receptors containing α 4- and δ -subunits to enhance tonic inhibition. In contrast, the sensitivity of GABA_A receptors mediating inhibition in the female γ 2^{Y365/367F} +/– to neurosteroids was markedly reduced compared with WT. We conclude that disrupting tyrosine phosphorylation of the γ 2-subunit activates a sex-specific increase in tonic inhibition, and this most likely reflects a genomic-based compensation mechanism for the reduced neurosteroid sensitivity of inhibition measured in female HT relay neurons.

Introduction

GABA-mediated inhibition requires the activation of GABA_A receptors (GABA_ARs) (Sieghart and Sperk, 2002). In the synaptic cleft, millimolar concentrations of GABA enable rapid, but brief phasic inhibition of postsynaptic neuronal excitability (Mody et al., 1994). While outside synapses, nanomolar GABA concentrations tonically activate extrasynaptic GABA_ARs, providing a persistent, less spatially restricted form of signaling (Otis et al., 1991; Mody, 2001). Both phasic and tonic inhibition are modified by changes to GABA release and uptake, and by alterations to GABA_AR numbers and their innate functional properties (Overstreet et al., 2000; Bianchi and Macdonald, 2002; Petrini et al., 2004; Luscher et al., 2011).

One important mechanism that modulates the efficacy of inhibition involves phosphorylation of GABA_ARs, which affects their function and trafficking (Moss and Smart, 1996; Brandon et al., 2003; Kittler et al., 2008; Abramian et al., 2010). Notably, clathrin-dependent endocytosis of GABA_ARs requires the interaction of β - and γ 2-subunits with the clathrin-adaptor protein, AP2. Phosphorylation of β (S409 in β 1–3, S410 in β 2) and γ 2-

subunits (Y365/367) by serine-threonine kinases and tyrosine kinases, respectively, regulates the GABA_AR-AP2 interaction thereby stabilizing receptors at inhibitory synapses (McDonald et al., 1998; Brandon et al., 2003; Kittler et al., 2008). Phosphorylation of the α 4-subunit (S443) can also promote the cell surface stability of GABA_ARs responsible for tonic inhibition (Abramian et al., 2010).

To gain insight into the role of GABA_AR phosphorylation in shaping the efficacy of synaptic inhibition, we used a γ 2 knock-in mouse in which the principal sites of tyrosine phosphorylation within the GABA_AR γ 2-subunit were mutated to phenylalanines (Y365/367F). Previously we noted that the Y365/367F homozygote is lethal and that male heterozygotes exhibited enhanced accumulation of synaptic GABA_ARs in the hippocampal CA3 region due to aberrant trafficking within the endocytic pathway. This enhanced inhibition correlated with a specific deficit in spatial object recognition, a behavioral trait that is associated with *regio* CA3 (Tretter et al., 2009). Here, we used this animal model to assess whether the tyrosine mutations affected the expression of α 4- and δ -subunits, both critical components of extrasynaptic GABA_ARs mediating tonic inhibition in several areas of the forebrain (Cope et al., 2005; Chandra et al., 2006). Indeed, little is known about how neurons coordinate and balance the distinct inhibitory modes of phasic and tonic.

We studied the dorsal lateral geniculate nucleus (dLGN), a part of the thalamic relay center important for the processing of visual information. In the dLGN, α 1 β γ 2 GABA_ARs are mainly responsible for phasic inhibition (Soltesz et al., 1990; Okada et al.,

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2000), while tonic inhibition depends on $\alpha 4\beta\delta$ receptors (Cope et al., 2005; Bright et al., 2007). Using patch-clamp recording, immunocytochemistry, and immunoblotting, we found that Y365/367F enhanced both phasic and tonic inhibition in female animals only. For tonic inhibition, preventing tyrosine phosphorylation of $\gamma 2$ -subunits resulted in a sex-specific up-regulation in the number of extrasynaptic GABA_ARs containing $\alpha 4$ - and δ -subunits, indicating that the long-term plasticity of phasic and tonic levels of GABA-mediated inhibition can be inter-related.

Materials and Methods

Animals. All procedures were performed in accordance with the UK Animals (Scientific Procedures) Act of 1986. Mice were housed under controlled conditions at 19–23°C, on a 12 h alternating light/dark cycle, in a ventilated room with access to food and water *ad libitum*. Mice homozygous for the Y365/367F mutation do not survive *in utero*, but heterozygotes continue into adulthood (Tretter et al., 2009). GABA_AR $\gamma 2$ Y365/367F heterozygous knock-in (Y365/367F +/–) mice and littermate controls were maintained on a C57BL/6J genetic background and had been backcrossed at least nine times. Resulting progeny were screened by PCR using ear notches taken at 2 weeks postnatal to determine genotype. The primers were as follows: forward primer: 5'-CAG GGT AAT TGC TAG GCC TGT TGG C-3'; reverse primer: 5'-CCA CAA TGT CAT CAA TGA GAT GAT G-3'. Mice were separated at weaning into male and female littermate groups.

Immunohistochemistry. Juvenile (P21–P28) male and female $\gamma 2$ knock-in mice (Y365/367F; Tretter et al., 2009) were anesthetized and intracardially perfused with saline solution followed by 4% paraformaldehyde. Brains were quickly removed, postfixed overnight, and cryoprotected in 30% sucrose. Sections (40 μ m; ~8 per brain) were cut using a freezing microtome and then incubated for 10 min in 0.1% H₂O₂ to block endogenous peroxidase activity. After washing in PBS, sections were incubated for 2 h in blocking solution (2% normal horse serum, 0.5% bovine serum albumin w/v, and 0.3% Triton X-100 in PBS) followed by incubation for 48 h at 4°C in blocking solution containing the primary polyclonal antibody against the $\alpha 4$ -subunit (1:1000; gift from W. Sieghart (Vienna); Bencsits et al., 1999). After rinsing in PBS, sections were incubated in blocking solution containing biotinylated anti-rabbit antibody for 2 h at room temperature, then rinsed in PBS and incubated for 1 h in ABC solution (Vectastain Elite ABC kit, Vector Laboratories). After washing, sections were incubated for 10 min in diaminobenzidine (peroxidase substrate) containing nickel chloride as an enhancing reagent. The reaction was terminated by washing twice in water. Sections were mounted onto slides, air-dried, dehydrated through graded alcohols followed by xylenes, and then mounted for viewing. Sections were visualized with an Olympus BX51 microscope (Olympus Optical). Optical density measurements were obtained from serial sections of wild-type (WT) and HT brains. The HT optical densities are normalized to the mean values obtained from WT brains. These measures were repeated in separate experiments for male and female mice.

Western blot analysis. dLGN was rapidly dissected out from coronal slices (250 μ m thick) obtained from juvenile (P21–P28) male and female $\gamma 2$ knock-in mice and homogenized in a buffer containing 150 mM NaCl, 50 mM Tris, pH 8.0, 5 mM EDTA, 1% nonyl phenoxypolyethoxylethanol, 0.5% sodium deoxycholate, 0.1% SDS, 1% phenylmethanesulfonyl fluoride in the presence of protease inhibitors (Thermo Scientific). The sample was rotated for 1 h at 4°C and then centrifuged at 16,100 $\times$ g for 15 min at 4°C; the supernatant was collected as the total protein fraction. Protein concentrations were determined using the BCA Protein Assay (Thermo Scientific). Either 20 μ g (δ -subunit) or 30 μ g ($\alpha 4$ -subunit) of total protein fraction was loaded onto a 10% SDS-polyacrylamide gel, subjected to electrophoresis and transferred to a pure nitrocellulose membrane. The membrane was blocked in 4% nonfat milk and probed with polyclonal antibodies specific for δ (1:1000) or $\alpha 4$ (1:4000; both gifts from W. Sieghart, Vienna) or monoclonal antibody specific for tubulin (1:1000). The blots were incubated with peroxidase-labeled anti-rabbit

or anti-mouse IgG (1:10,000) and immunoreactive proteins were visualized using enhanced chemiluminescence. Optical density was determined using ImageJ software. Tubulin was used as a loading control.

Slice preparation. Coronal thalamic slices (250 μ m thick) were cut in a slicing solution composed of the following (in mM): 130 K-gluconate, 15 KCl, 0.05 EGTA, 20 HEPES, 4 Na-pyruvate, 25 glucose, and 2 kynurenic acid, pH to 7.4 with NaOH, bubbled with 95% O₂ and 5% CO₂ using a Leica VT1200S Vibratome slicer. Slices were incubated at 37°C for 1 h, during which the gluconate slicing solution was gradually replaced with artificial CSF (aCSF) containing the following (in mM): 125 NaCl, 26 NaHCO₃, 2.5 KCl, 1 MgCl₂, 1.25 NaH₂PO₄, 2 CaCl₂, 25 glucose, and 2 kynurenic acid. Slices were then maintained at room temperature in this solution, bubbled with 95% O₂/5% CO₂, until required.

Electrophysiology. Thalamic neurons in the dLGN were visually identified using a Nikon Eclipse FN1 microscope equipped with a 40 $\times$ immersion objective and a video camera. Whole-cell voltage-clamp recordings were performed at room temperature using an Axopatch 200B amplifier (Molecular Devices). Patch pipettes (thin-walled borosilicate glass, GC150TF-10; Harvard Apparatus) had resistances of 3–5 M Ω when filled with intracellular solution containing the following (in mM): 140 CsCl, 2 NaCl, 10 HEPES, 5 EGTA, 2 MgCl₂, 0.5 CaCl₂, 2 Na-ATP, 2 QX-314, and 5 sucrose; pH 7.3 with 1 M CsOH. Bicuculline, 3 α ,5 α -tetrahydrodeoxycorticosterone (THDOC), 4,5,6,7-tetrahydroisothiazolo-[5,4-c]pyridin-3-ol (THIP), and zinc chloride were added to the aCSF. Sodium orthovanadate (Na₃VO₄, 100 μ M) was included in the electrode solution in some experiments.

Data acquisition and analysis. Membrane currents were low-pass filtered at 5 kHz and acquired at 20 kHz. Series resistance was compensated by ~40% and data discarded if this increased by ~25%. Currents and variances were analyzed off-line using WinEDR and WinWCP (John Dempster, University of Strathclyde, UK). To determine average holding currents, a continuous current recording was measured for a 60 s epoch sampled every 1 s, discarding those coincident with spontaneous IPSCs (sIPSCs). A drug effect on the holding current was defined by the difference between average holding currents in control and during drug application. All-point histograms were constructed for the holding current taking data samples before and after bicuculline (20 s) and fitted with a single Gaussian distribution function of the following form:

$$f(x) = A \times \frac{e^{-(x-\mu)^2/2\sigma^2}}{\sigma\sqrt{2\pi}} + C$$

The fit was constrained symmetrically around the peak frequency to avoid any bias caused by the presence of sIPSCs. A defines the amplitude and C is a constant defining the pedestal of the histogram. This function provided the Gaussian mean baseline current (μ) and SD (σ). The baseline noise (root mean square, rms) was also calculated before and during bicuculline treatment. To estimate average baseline noise, a continuous current recording was analyzed over a 30 s epoch and sampled every 100 ms; in the case of control recordings (during which sIPSCs are present), the median was then calculated every 5 s and values > 2 SDs outside the median (greater variation reflecting sIPSCs) were discarded. Baseline GABA-mediated current noise was defined as the difference between rms values before and after bicuculline. sIPSCs were detected using amplitude- and kinetics-based criteria with MiniAnalysis Program 6.0.7 (Synaptosoft). Frequency was calculated using events detected over 60 s epochs. At least 100 uncontaminated sIPSCs were used to construct averaged sIPSC waveforms and to estimate kinetic parameters.

Expression of GABA_A recombinant receptors and electrophysiology. HEK293 cells were transfected using a calcium phosphate protocol. Equal amounts of cDNAs encoding GABA_AR subunits and enhanced green fluorescent protein were used (4 μ g total per dish). Cells were used for electrophysiology 16–24 h post-transfection. Cells were continuously perfused at room temperature with Krebs solution containing the following (in mM): 140 NaCl, 4.7 KCl, 1.2 MgCl₂, 2.52 CaCl₂, 11 glucose, and 5 HEPES, adjusted to pH 7.4 with 1 M NaOH. Patch pipettes (3–4 M Ω) were filled with an intracellular solution containing the following (in mM): 1 MgCl₂, 120 KCl, 11 EGTA, 10 HEPES, 1 CaCl₂, and 2 adenosine triphosphate, adjusted to pH 7.2 with 1 M NaOH. Na₃VO₄ (100 μ M) and active Src kinase (1.44 $\times 10^{-3}$ U/ml; Merck Millipore) were included in

the electrode solution in some experiments. Whole-cell GABA activated currents were recorded from HEK cells clamped at -10 mV, filtered at 5 kHz, and digitized at 50 kHz. GABA- and THDOC-containing solutions were locally applied via a U-tube application system (Mortensen and Smart, 2007); the duration of GABA application was 2 s. Amplitudes of whole-cell GABA responses were measured and normalized to the maximum response evoked by saturating concentrations of GABA. Each dose–response curve to GABA was curve fitted using the following Hill equation:

$$\frac{Y}{Y_{\max}} = \frac{[A]^n}{[A]^n + EC_{50}^n}$$

Y is the peak response to GABA, $Y_{\max}$ represents the maximum peak response to saturating concentrations of GABA, EC_{50} is the concentration of GABA, $[A]$, producing 50% of the maximal response, and n is the Hill coefficient.

Statistical analysis. Data are expressed as mean $\pm$ SEM. Tests were performed using Origin 6.0 (OriginLab). A Shapiro–Wilk test determined whether data were normally distributed and, if so, differences between two groups were examined using paired and unpaired Student's t test. Concerning association analysis, statistical significance was assessed with the Pearson product moment correlation coefficient. Differences between more than two groups were assessed using ordinary and repeated-measures ANOVA and nonparametric methods with *post hoc* tests, as appropriate.

Results

Sex-specific effect of $\gamma 2^{Y365/367F}$ on $\alpha 4$ -subunit expression levels

To investigate how phosphorylation of GABA_AR subunits can allow neurons to regulate GABA-mediated inhibition, we used our mouse knock-in for which residues Y365 and Y367 in the GABA_AR $\gamma 2$ -subunit have been mutated to phenylalanines (Y365/367F). In principle, this substitution should prevent receptor internalization and therefore potentiate phasic inhibition (Kittler et al., 2008; Tretter et al., 2009), but the consequences for tonic inhibition are unknown.

First, we used immunohistochemistry to assess the impact of switching residues on the expression levels of GABA_AR subunits that mediate tonic inhibition. The $\alpha 4$ -subunit was selected because of its involvement in extrasynaptic receptors and tonic inhibition in the dentate gyrus (DG) as well as specific thalamic nuclei (Cope et al., 2005; Chandra et al., 2006; Bright et al., 2007; Shen et al., 2007). We compared $\alpha 4$ -subunit expression levels in the brains of age-matched Y365/367F heterozygotes (HT) and WT littermates (homozygotes are not viable *in utero*; Tretter et al., 2009). While in the HT males $\alpha 4$ -subunit expression was reduced in the DG ($55.4 \pm 6.4\%$, $n = 5$, $p < 0.01$) and thalamus ($62.5 \pm 9.2\%$, $n = 5$, $p < 0.01$) compared with WT (100%; Fig. 1A,C), $\alpha 4$ expression was raised in the same brain regions for HT females (DG: $164.8 \pm 11.2\%$, $n = 6$, $p < 0.01$; thalamus: $195.4 \pm 19.2\%$, $n = 6$, $p < 0.01$), potentially affecting the level of tonic inhibition (Fig. 1B,C).

Figure 1. The $\alpha 4$ -subunit expression levels are increased in a sex-specific manner in HT ($\gamma 2^{Y365/367F} +/ -$) mice. **A, B**, Whole brain sagittal sections (40 μ m) from male (**A**) and female (**B**) WT and HT mice were immunostained for $\alpha 4$ -subunits followed by horseradish peroxidase-conjugated secondary antibody. Arrows indicate the DG (upper) and the thalamus (lower). **C**, Bar graphs for the mean $\alpha 4$ optical density measured in male and female HT, both thalamus and DG, compared with WT (= 100%; $n = 5$ for male DG and thalamus; $n = 6$ for female DG and thalamus); ** $p < 0.01$.

Figure 2. Expression of GABA_AR $\alpha 4$ - and δ -subunits in $\gamma 2$ knock-in dLGN. **A, B**, Left, Immunoblot analysis of $\alpha 4$ (**A**) and δ (**B**) protein (30 μ g/lane or 20 μ g/lane, respectively) in total homogenates prepared from the dLGN of male ($n = 3$) and female ($n = 10$; $\alpha 4$) and female only ($n = 7$; δ) mice of the same age. Tubulin is used as an internal standard. Molecular weight markers are shown. **A, B**, Right, Bar graphs reporting mean $\alpha 4$ (**A**) and δ (**B**) expression in HT dLGN compared with female WT. The optical density of bands is corrected according to the tubulin standard and normalized to female WT levels (= 100%, * $p < 0.05$).

$\alpha 4$ - and δ -subunits expression in female $\gamma 2^{Y365/367F} +/ -$

Since $\alpha 4\beta\delta$ receptors are considered to mediate tonic inhibition in the dLGN (Cope et al., 2005; Bright et al., 2007), we used Western blot analysis to investigate $\alpha 4$ - and δ -subunits relative expression levels in $\gamma 2$ knock-in animals (Fig. 2A,B). In female and male WT and HT animals, dLGN total protein fractions were immunoblotted with anti- $\alpha 4$ antibody, with

Figure 3. $\gamma 2^{Y365/367F}$ does not affect inhibition in the male dLGN. **A**, Representative currents recorded at a holding potential of -60 mV from dLGN relay neurons in WT (left) and HT (right). Bath-applied bicuculline ($20 \mu\text{M}$; gray bar) blocks inward sIPSCs and reveals an outward shift in the holding current and a reduction in rms baseline noise. The holding currents before and after bicuculline are shown as dotted lines. The Gaussian curves depict the mean current and its SD neglecting the distortion caused by the sIPSCs (see Materials and Methods). **B**, Bar graphs of mean holding currents in aCSF, after bicuculline (Bic) and the resulting GABA tonic current for WT ($n = 11$) and HT dLGN relay neurons ($n = 8$; $p = 0.778$). **C**, Bar graphs of mean rms noise in aCSF, after bicuculline and the resulting GABA current noise in WT ($n = 11$) and HT dLGN relay neurons ($n = 8$; $p = 0.961$). **D**, **E**, sIPSC frequency (**D**) and amplitude (**E**) are similar in male WT and HT dLGN relay neurons ($p = 0.086$ and $p = 0.930$, respectively). **F**, **G**, sIPSC rise (**F**) and decay (**G**) times are comparable between male WT and HT dLGN relay neurons ($p = 0.073$ and $p = 0.730$, respectively).

tubulin levels used as a loading control (Fig. 2A). Comparing $\alpha 4$ -subunit-immunoreactive bands revealed a significant increase in female HTs compared with WT (Kruskal–Wallis test with Dunn’s multiple-comparison post-test, $p < 0.05$; $n = 10$ age-matched, female WT and HT pairs). For male WT and HTs, there was a tendency toward decreased $\alpha 4$ abundance in male HTs (Fig. 2A; Dunn’s multiple-comparison post-test, $p > 0.05$, $n = 3$ age-matched, male WT and HT pairs).

Given that $\alpha 4$ -subunits are frequently coassembled with δ -subunits in extrasynaptic GABA_ARs (Sur et al., 1999; Jia et al., 2005), we also evaluated δ -subunit expression levels in female WT and HT animals (Fig. 2B). Western blots revealed significantly increased expression of δ -subunits in HT mice compared with WT ($143 \pm 16\%$, $n = 7$, age-match WT and HT pairs, $p < 0.05$; Fig. 2B).

Thus, both immunoblotting and immunohistochemistry provided evidence of a sex-specific effect of the $\gamma 2^{Y365/367F}$ mutation on $\alpha 4$ -subunit expression levels in the thalamus, together with upregulated extrasynaptic δ -subunit levels in the dLGN of female $\gamma 2^{Y365/367F} +/ -$, potentially affecting the level of tonic inhibition.

Tonic and phasic inhibition in male dLGN for $\gamma 2^{Y365/367F} +/ -$ is unaffected

Given the distinct difference in $\alpha 4$ and δ expression levels, we first examined GABA-mediated tonic current in thalamic relay neu-

rons of the male dLGN. These cells display a tonic GABA_A-mediated conductance that relies on extrasynaptic $\alpha 4$ - and δ -subunits (Cope et al., 2005), and on vesicular GABA release (Bright et al., 2007), while phasic inhibition involves $\gamma 2$ -subunit-containing GABA_ARs. Using whole-cell recording in acute thalamic slices under control conditions (in the presence of 2 mM kynurenic acid), bicuculline ($20 \mu\text{M}$) blocked the sIPSCs and clearly reduced both the holding current and rms baseline noise. This revealed a tonically active GABA current (in the absence of applied GABA), in accord with previous reports (Cope et al., 2005; Bright et al., 2007; Fig. 3A). Comparing male WT and HT relay neurons, there were no differences in the holding current (WT tonic current -49.7 ± 11.4 pA, $n = 11$; HT -54.1 ± 8.6 pA, $n = 8$; $p = 0.778$; Fig. 3A,B) and rms baseline noise (WT 1.5 ± 0.2 pA; HT 1.5 ± 0.4 pA; $p = 0.961$; Fig. 3A,C). Comparing the properties of sIPSCs in the same relay neurons of WT and HTs in male mice revealed similar frequencies (Fig. 3D), mean sIPSC amplitudes (Fig. 3E), and rise (Fig. 3F) and decay times (Fig. 3G). Therefore, for male dLGN relay neurons from $\gamma 2^{Y365/367F}$ mice, there were no obvious effects of preventing tyrosine phosphorylation of the $\gamma 2$ -subunit on either tonic or phasic inhibition.

Increased tonic inhibition in female dLGN of $\gamma 2^{Y365/367F} +/ -$

Periodic alterations in specific GABA_A subunits can occur during the estrous cycle in mice, causing cyclic changes to tonic inhibition in hippocampal neurons. In particular, during late diestrus (high-progesterone phase), enhanced expression of GABA_A δ -subunits led to an increased tonic inhibition (Maguire et al., 2005). To avoid estrous cycle variations confounding our studies, and to allow comparison to results from juvenile males, recordings were made from juvenile female mice. For WT dLGN neurons of female mice, the mean holding current (-131.6 ± 18.6 pA in aCSF) was reduced by $20 \mu\text{M}$ bicuculline (-116.9 ± 18.8 pA; Fig. 4A), while the rms baseline noise was 4.0 ± 0.3 and 3.4 ± 0.2 pA, respectively ($n = 20$; Fig. 4A). In comparison, for HT relay cells, a mean holding current of -117.7 ± 14.2 pA in aCSF was reduced by bicuculline to -92.3 ± 12.6 pA (Fig. 4B); with rms baseline noise of 5.0 ± 0.3 and 4.0 ± 0.3 pA, respectively ($n = 26$; Fig. 4B). Both the tonic GABA current (-25.4 ± 3.2 pA) and current noise (1.0 ± 0.2 pA; Fig. 4B) were significantly augmented in female HT relay cells compared with WT (-14.7 ± 1.8 and 0.6 ± 0.1 pA, respectively; $p < 0.05$; Fig. 4A), indicating a sex-specific effect of $\gamma 2^{Y365/367F}$ on GABA-mediated tonic inhibition. Using Kruskal–Wallis and Dunn’s multiple-comparison tests to compare tonic current across all conditions (female and male, WT and HT), we found a significant increase in female HTs compared with WT ($p < 0.05$), and for males, tonic currents in both WT and HTs were increased compared with WT females ($p < 0.01$).

Figure 4. $\gamma 2^{Y365/367F}$ increases tonic inhibition in female dLGN. **A, B**, Top, Currents recorded from WT (**A**) and HT (**B**) dLGN relay neuron at -60 mV. Application of bicuculline ($20 \mu\text{M}$, bar) blocks the tonic conductance and reduces the background noise. Note female HT relay neurons show increased tonic GABA current and noise. Graphs show holding currents (middle) and rms baseline noise values (bottom) for all cells recorded in female WT (left; $n = 20$) and HT (right; $n = 26$) mice. Values in aCSF are shown in red and after bicuculline application in blue, while the resulting GABA_A -mediated tonic current is shown in black. Respective mean values are in the same color, shifted to the right. Insets to graphs represent an enlargement of tonic values. Both HT mean tonic current and GABA_A -mediated current noise are significantly augmented. $*p < 0.05$ compared with WT.

Phasic inhibition in female dLGN $\gamma 2^{Y365/367F} +/ -$ is potentiated

Tonic activation of dLGN δ -subunit GABA_A Rs is sensitive to the overall level of inhibition being dependent upon spillover from the presynaptic GABA release. Indeed, the tonic conductance was abolished when transmitter release probability was reduced or action potential-evoked release was blocked (Bright et al., 2007). To explore whether the increased tonic inhibition in female $\gamma 2^{Y365/367F} +/ -$ might rely on enhanced release of presynaptic GABA, we analyzed sIPSC frequencies in female WT and HT dLGN relay neurons. This was augmented from 4.5 ± 0.4 Hz (WT) to 7.1 ± 0.7 Hz (HT) ($n = 20$ – 26 ; $p < 0.01$; Fig. 5*A, B*).

The distribution of all interevent intervals for sIPSCs in WT and HT relay neurons was described by the sum of three Gaussian components. The means for all three Gaussian populations were reduced indicating shorter intervals between sIPSCs recorded from HT dLGN relay neurons (Fig. 5*B*). The sIPSC mean amplitude was also significantly increased from 29.5 ± 1.5 pA (WT) to 36.1 ± 2.5 pA (HT) ($n = 20$ – 26 ; $p < 0.05$; Fig. 5*A*) and this could have apparently increased sIPSC frequency following the increased resolution of small, previously undetected, amplitude events. Examining the sIPSC amplitude distributions revealed three distinct populations with an increased proportion of higher amplitude sIPSCs for the HTs (Fig. 5*C*).

Analyzing the sIPSC kinetics in the respective genotypes revealed that rise and decay times were comparable between WT and HT female dLGN relay neurons ($p = 0.835$ and $p = 0.355$, respectively; Fig. 5*D, E*). Thus, in female neurons, the $\gamma 2^{Y365/367F}$ mutation increased only the mean sIPSC amplitude and frequency.

Assessing sIPSC parameters across all conditions (female and male, WT and HT) with either ANOVA (rise and decay times) or

Figure 5. Synaptic inhibition in dLGN relay neurons of female WT and $\gamma 2^{Y365/367F} +/ -$ mice. **A**, Recordings of sIPSCs obtained from WT and HT dLGN relay neurons held at -60 mV in the presence of 2 mM kynurenic acid at room temperature. Inset depicts average waveforms (see Materials and Methods) for these cells (WT, black; HT, gray). Note increased sIPSC amplitude and unchanged kinetics in HT cells. **B, C**, Analysis of sIPSC interevent intervals (**B**) and amplitudes (**C**) for WT and HT dLGN relay neurons taken from thalamic slices of juvenile female mice. Histograms are fitted with the sum of three Gaussian distributions (2000 events per genotype) in WT and HT mice. Arrow indicates the mean of the largest amplitude events in HTs, a population infrequently found in WT. **D, E**, Mean rise time (**D**) and decay time (**E**) for sIPSCs from female WT and HT dLGN relay neurons.

Kruskal–Wallis tests (frequency and amplitude), as appropriate, revealed that mean sIPSC frequency and amplitude in female HT were increased compared with WT. In addition, for both male WT and HT, sIPSC frequencies were significantly augmented compared with female WT ($p < 0.05$ and $p < 0.01$, respectively). This increased frequency may account for the larger tonic currents measured in males.

Coincidence of phasic and tonic inhibition in female dLGN relay neurons

To better understand the interplay between phasic and tonic inhibition in female WT and HT mice, we plotted individual tonic currents measured in WT and HT dLGN relay neurons against the mean sIPSC frequencies and amplitudes, measured in the same cells. For WT there was no relation between tonic currents and relative sIPSC frequencies ($r = 0.032$, $p = 0.888$) (Fig. 6*A*), while for HT relay neurons, a linear relationship was evident ($r = 0.567$, $p < 0.05$; Fig. 6*B*). By plotting individual tonic currents against mean sIPSC amplitudes, a poor relationship was again evident for WT relay cells ($r = 0.369$, $p = 0.108$; Fig. 6*A*), while a clear linear relationship was manifest for female HTs ($r = 0.550$, $p < 0.05$; Fig. 6*B*).

Figure 6. Relationship between tonic and phasic inhibition in female dLGN. Plots of mean GABA_A-mediated tonic currents against mean sIPSC frequencies (top) or amplitudes (bottom) for all cells examined from female WT (**A**) and HT animals (**B**). Positive correlations between the tonic current and both the mean sIPSC frequency and amplitude are only present in HT dLGN relay neurons ($p < 0.05$).

Increased THIP-induced current in female HT dLGN relay neurons

The higher levels of tonic inhibition in female HT dLGN relay neurons would be accounted for by the increased expression of GABA_A $\alpha 4$ - and δ -subunits compared with WT. The presence of the δ -subunit confers unique pharmacological properties on GABA_ARs including a high sensitivity to the superagonist THIP (Brown et al., 2002; Cope et al., 2005; Mortensen et al., 2010). To determine the subunit nature of GABA_ARs underpinning the tonic current in female WT and HT neurons, 1 μ M THIP, a concentration that is selective for GABA_ARs containing the δ -subunit was applied (Brown et al., 2002; Jia et al., 2005; Mortensen et al., 2010). THIP rapidly activated an inward current over 3 min and increased current noise for both WT and HT thalamic neurons in a reversible manner (Fig. 7*A,B*). For female WT neurons, the THIP-induced inward current was -163.3 ± 28.4 pA (Fig. 7*A*), which was significantly augmented in female HT neurons (-266.2 ± 31.6 pA; $n = 11$, $p < 0.05$; Fig. 7*B*). Taken overall, this suggests that an increased number of extrasynaptic δ -subunit-containing GABA_ARs in HT females could underpin the higher level of tonic inhibition displayed by these animals.

For male WT dLGN neurons, the THIP-induced inward current (-168.4 ± 11.5 pA; $n = 6$) was no different to that measured in dLGN relay neurons of WT females ($p = 0.885$). This indicates that there are similar extrasynaptic $\alpha 4\beta\delta$ GABA_AR levels in the thalamus of WT female and male animals and possibly the higher tonic current in males is likely to be due to presynaptic GABA release increasing ambient GABA levels.

Increased Zn²⁺-block of tonic inhibition in female $\gamma 2^{Y365/367F}$ +/-

Micromolar concentrations of Zn²⁺ have little effect on $\alpha 1\beta 2/3\gamma 2$ GABA_ARs, which are mainly responsible for phasic inhibition in the thalamus (Draguhn et al., 1990; Smart and Constanti, 1990; Soltesz et al., 1990; Smart et al., 1991, 1994; Okada et al., 2000; Pirker et al., 2000; Sassòe-Pognetto et al., 2000), but substantially inhibit current mediated by GABA_ARs containing the δ -subunit (Saxena and Macdonald, 1994; Krishek et al., 1998).

Figure 7. THIP currents in female WT and HT dLGN relay neurons. Current traces from WT (**A**, top) and HT (**B**, top) dLGN relay neurons at -60 mV. Bath-applied THIP (bar) enhances tonic GABA current and associated baseline noise in WT and to a greater extent in HT dLGN relay neurons. **A, B**, Bottom, Graphs show individual holding current values in aCSF (left symbols), after THIP application (middle symbols), and the resulting THIP-induced inward current (ΔI , right symbols) for all cells examined from WT ($n = 8$) and HT animals ($n = 11$). Respective mean values are shifted to the right. Note mean THIP-induced inward current is significantly increased in HT compared with WT ($*p < 0.05$).

Figure 8. Zn²⁺-sensitive currents in female $\gamma 2^{Y365/367F} +/-$ dLGN. **A, B**, Current recordings from WT (**A**) and HT dLGN relay neurons (**B**) under control conditions and during the application of 30 μ M Zn²⁺ (gray bar) and then 20 μ M bicuculline (light gray bar). Bicuculline block is similar between WT and HT relay neurons, while the block induced by Zn²⁺ is increased in the HT. **C**, Bar graphs quantifying the change in holding current caused by 30 μ M Zn²⁺ and 20 μ M bicuculline, in WT ($n = 6$) and HT ($n = 5$) cells. Note the mean Zn²⁺ block is significantly augmented in female HT dLGN relay neurons ($*p < 0.05$ compared with WT); in contrast, bicuculline-induced block is comparable between WT and HT relay neurons. **D**, Histograms demonstrating that 30 μ M Zn²⁺ does not affect phasic inhibition, with mean sIPSC amplitude unchanged between aCSF and Zn²⁺ for both WT ($p = 0.477$) and HT cells ($p = 0.937$).

To corroborate our observation that extrasynaptic GABA_ARs containing the δ -subunit are upregulated in the thalamus of female HTs, we treated both WT and HT dLGN neurons with 30 μ M Zn²⁺, a concentration selective for δ -subunit-containing receptors (Smart et al., 1994; Jia et al., 2005; Fig. 8*A,B*). We subsequently applied 20 μ M bicuculline to compare the Zn²⁺-induced

block with that caused by bicuculline (Fig. 8 *A,B*). Analyzing female WT dLGN relay cells revealed that the mean outward current (tonic block) induced by Zn^{2+} was 10.9 ± 1.8 pA, while the residual bicuculline block was 7.9 ± 1.9 pA ($n = 6$; Fig. 8 *A,C*). In contrast, for female HT neurons, the mean values were 21.7 ± 4.3 pA (+ Zn^{2+}) and 3.5 ± 1.3 pA (+ bicuculline) ($n = 5$; Fig. 8 *B,C*). The block induced by Zn^{2+} was significantly increased in female HT relay cells ($p < 0.05$), while the residual bicuculline block was comparable between the two genotypes ($p = 0.073$) (Fig. 8C). These results are in accord with a higher number of extrasynaptic δ -subunit-containing receptors in the dLGN of female HTs. To exclude the possibility of Zn^{2+} blocking synaptic GABA_ARs, the mean sIPSC amplitudes in control and in Zn^{2+} for both WT and HT dLGN neurons were compared (Fig. 8D), and revealed no effect of Zn^{2+} .

Neurosteroid modulation of sIPSCs in female $\gamma 2^{365/367\text{F}}$ +/– is unaffected

Given the sex specificity of $\gamma 2^{365/367\text{F}}$ on phasic and tonic inhibition in the thalamus, we explored the sensitivity of female WT and HT dLGN neurons to THDOC, a naturally occurring neurosteroid. Neurosteroids potently regulate neuronal excitability by modulating GABA_ARs (Belelli and Lambert, 2005). In addition, neurosteroids also regulate GABA_ARs over a longer timescale by altering the expression of specific GABA_AR subunits during the estrous cycle, during pregnancy, and in numerous disorders (Maguire et al., 2005). By applying THDOC (100 nM) to female WT and HT dLGN relay neurons, similar effects were recorded on sIPSC rise and decay times (Fig. 9 *A,B*). However, THDOC increased mean sIPSC amplitude and frequency in female WT neurons with no effect on these parameters in HT cells (Fig. 9 *A,B*).

To investigate why HT neurons seemed unaffected, THDOC modulation was studied in HEK293 cells expressing recombinant WT or mutant GABA_AR subunits: $\alpha 1\beta 2\gamma 2$ or $\alpha 1\beta 2\gamma 2^{365/367\text{F}}$ to simulate WT and HT conditions. GABA concentration–response curves for WT and mutant receptors were similar (WT: $\text{EC}_{50} = 15.06 \pm 1.70$ μM , Hill coefficient = 1.05 ± 0.08 , $n = 4$; mutant: $\text{EC}_{50} = 12.65 \pm 2.93$, Hill coefficient = 1.09 ± 0.09 , $n = 4$; $p = 0.50$ and $p = 0.79$ for EC_{50} and Hill coefficient, respectively). Next, we applied 2 μM GABA (EC_{10}) every 2 min before applying at 15 min, 100 nM THDOC to potentiate the GABA current. The analysis revealed a similar potentiation at WT and mutant receptors (WT: 2.68 ± 0.28 , $n = 6$; $\gamma 2^{365/367\text{F}}$: 2.26 ± 0.26 , $n = 6$; $p = 0.29$; control GABA response = 1; Fig. 9C).

To ascertain whether the lack of effect of THDOC on HT GABA responses was a result of altered tyrosine phosphorylation state of the $\gamma 2$ -subunit, we examined the effects of Src (1.44×10^{-3} U/ml), the physiologically active form of the tyrosine kinase responsible for $\gamma 2^{365/367}$ phosphorylation (Moss et al., 1995), and Na_3VO_4 (100 μM), which inhibits tyrosine phosphatases (Gordon, 1991). Using recombinant WT ($\alpha 1\beta 2\gamma 2$) and $\gamma 2$ mutant receptors expressed in HEK cells, intracellular dialysis of activated Src increased the amplitude of GABA currents for WT $\alpha 1\beta 2\gamma 2$ receptors, but not mutants, as reported previously (Moss et al., 1995). After 15 min, we coapplied 100 nM THDOC; however, no difference was observed in the THDOC potentiation between WT and mutant-expressing cells (WT + Src: 2.44 ± 0.25 , $n = 4$; $\gamma 2$ Y365/7F + Src: 2.62 ± 0.33 , $n = 7$; $p = 0.72$) (Fig. 9C).

These results based on heterologous expression studies suggest that the inability of THDOC to potentiate synaptic activity in HT compared with WT relay neurons is not due to a change in the

tyrosine phosphorylation status of the receptor, nor any unforeseen structural change induced by the $\gamma 2$ -subunit mutation.

Neurosteroid modulation of dLGN sIPSCs is sex specific and dependent on GABA_AR phosphorylation state

To explore neurosteroid modulation of sIPSCs in female WT and HT thalamic neurons, we manipulated tyrosine phosphorylation levels in female WT dLGN relay cells by adding 100 μM Na_3VO_4 to the pipette solution. After 10 min, Na_3VO_4 significantly increased mean sIPSC amplitude and frequency, without affecting rise and decay times (Fig. 10 *A,B*). Subsequent bath application of 100 nM THDOC resulted in sIPSCs rise and decay times being increased, but the enhanced GABA_AR phosphorylation state promoted by Na_3VO_4 prevented any increase in the sIPSC amplitude or frequency, a result similar to that observed with female HT dLGN relay neurons, suggesting an interaction between GABA_AR phosphorylation and THDOC modulation of sIPSCs (Fig. 10 *A,B*).

We then studied the sensitivity of male WT dLGN relay neurons to THDOC. Comparing synaptic activity in aCSF (= 100%) and after 100 nM THDOC revealed augmented mean rise ($130 \pm 6\%$, $p < 0.01$) and decay times ($143 \pm 6\%$, $p < 0.001$) for sIPSCs, accompanied by no changes either in amplitude ($130 \pm 13\%$, $p = 0.052$) or in frequency ($134 \pm 17\%$, $p = 0.086$) although a tendency to increase was evident.

Discussion

This study shows that by mutating two tyrosine residues, Y365F and Y367F, which are substrates for tyrosine phosphorylation in the GABA_AR $\gamma 2$ -subunit, both phasic and tonic GABA-mediated inhibition by $\gamma 2$ - and δ -subunit-containing GABA_ARs are promoted in a sex-specific manner in the thalamus. Immunostaining indicated that $\alpha 4$ expression levels were raised in female HT DG and thalamus, and decreased in the same areas of male brains. In male dLGN there was no difference in phasic and tonic inhibition between WT and HT mice contrasting with the increased frequency and amplitude of sIPSCs, together with augmented THIP- and Zn^{2+} -sensitive currents in HT relay neurons from female dLGN. Immunoblotting suggested the increased tonic current in female HT mice was probably due to upregulated expression of δ -subunits in conjunction with $\alpha 4$. Thus, tyrosine phosphorylation at $\gamma 2$ -subunits is critically important for both phasic and tonic inhibition. In terms of sensory processing in the thalamus, increased inhibition in this area, which is vital for the processing of visual information, could result in increased visual contrast (Wang et al., 2011). Enhanced GABA inhibition could also impact on neural responses by affecting the membrane potential and thus the extent of tonic and burst firing modes of thalamic relay cells (Wang et al., 2011).

Modulation of GABA_ARs by phosphorylation

GABA_ARs are modulated by a number of serine/threonine and tyrosine kinases that affect receptor function and/or cell surface expression patterns. The kinase substrates are GABA_AR $\alpha 4$ -, $\beta 1$ –3- and $\gamma 2$ -subunits (Moss et al., 1995; McDonald et al., 1998; Houston et al., 2009; Abramian et al., 2010). In the $\gamma 2$ -subunit, the major tyrosine phosphorylation sites Y365 and Y367 are incorporated into the binding motif for AP2, a crucial protein recruiting cell-surface cargo for clathrin-coated endocytic vesicles (Schmid, 1997). Phosphorylation of these $\gamma 2$ -subunit tyrosines reduces their ability to bind AP2 in striatal neurons. Furthermore, disrupting this interaction increased the number of recep-

Figure 9. Differential THDOC modulation of synaptic activity in female $\gamma 2^{Y365/367F} +/ -$ dLGN relay neurons. **A**, Recordings of sIPSCs from WT and HT dLGN relay neurons at -60 mV in aCSF (left) and after 100 nM THDOC (right). **B**, Analysis of sIPSC amplitude (top left), frequency (top right), rise time (bottom left), and decay time (bottom right) for WT (filled bars) and HT (open bars) dLGN relay neurons in aCSF or during THDOC treatment (values in aCSF are defined as 100%) ($*p < 0.05$ comparing aCSF and THDOC data). **C**, Bar graph of the potentiation of $2 \mu\text{M}$ GABA responses by 100 nM THDOC for recombinant $\alpha 1\beta 2\gamma 2\text{L}$ (WT) and $\alpha 1\beta 2\gamma 2^{Y365,367F}$ GABA_ARs ($\gamma 2^{Y365/367F}$), in the absence or presence of the active form of the tyrosine kinase Src.

Figure 10. Tyrosine phosphorylation and THDOC modulation of synaptic activity in female WT dLGN relay neurons. **A**, Recordings of sIPSCs from female WT dLGN relay neurons at -60 mV internally exposed to Na_3VO_4 ($100 \mu\text{M}$) at $t = 0$ and 10 min, and after 100 nM THDOC. **B**, sIPSC amplitude and frequency were enhanced after 10 min of Na_3VO_4 but not after THDOC; sIPSC rise and decay times were unaffected by Na_3VO_4 , but significantly increased by THDOC (all values at $t = 0$ are defined as 100%) ($n = 5$, $*p < 0.05$, Friedman test and Dunn's multiple-comparisons test).

tors expressed on the cell surface accompanied by an enhanced mIPSC amplitude (Kittler et al., 2008).

We created a knock-in mouse line in which Y365 and Y367 are mutated to phenylalanines to examine the significance of tyrosine phosphorylation for network behavior; the resulting animal model provided clear evidence that GABA_A receptor phosphorylation regulates the efficacy of synaptic inhibition by modulating receptor trafficking in the endocytic pathway in the hippocampus (Tretter et al., 2009). Our findings in the present study demonstrate that in the $\gamma 2^{Y365/367F} +/ -$ line synaptic activity is also enhanced in the dLGN, consistent with increased GABA_A receptor stability at inhibitory synapses in HT mice. However, quite unexpectedly, we also observed a clear increase in tonic inhibition in $\gamma 2^{Y365/367F} +/ -$ thalamic relay neurons, suggesting a combined upregulation of synaptic and tonic inhibitory tone. Interestingly, the effect of Y365/367F on phasic and tonic GABA-mediated inhibition was sex-specific and limited to females.

Phasic and tonic GABA inhibitory modes

Between the GABA_ARs anchored at synapses and those located in extrasynaptic domains there is a molecular, physiological, and topographical diversity. Although neuronal excitability is determined by both phasic and tonic inhibition, little is known about how neurons coordinate these two distinct inhibitory modes. Some details come from the use of GABA receptor subunit knock-out lines, but carry the inherent problem of compensation. In the adult $\delta -/ -$ mouse, increased $\gamma 2$ -subunit expression is apparent together with a reduction in $\alpha 4$ -subunit levels (Peng et al., 2002). These changes were traced to brain areas that normally expressed the δ -subunit, suggesting potential compensation. In cerebellar granule cell slice recordings from $\alpha 6 -/ -$ mice the tonic GABA conductance was absent, but normal IPSC frequency was retained. The excitability of these cells was maintained by an increased voltage-independent K^+ leak conductance (Brickey et al., 2001). In contrast, $\alpha 4 -/ -$ mice lacked tonic inhibition in both hippocampal dentate granule cells and thalamic relay neurons, but caused a diverse impact on synaptic activity (Chandra et al., 2006). The Y365/367F mutation characterized in our study induced a sex-specific increase in both phasic and tonic inhibition.

Increased tonic current in female $\gamma 2^{Y365/367F}$ and extrasynaptic $\alpha 4/\delta$ GABA_ARs

Previous studies have established that tonic inhibition in the dLGN is most likely mediated by $\alpha 4\beta\delta$ -subunit assemblies (Sur et al., 1999; Cope et al., 2005). We measured an increase in both tonic inhibition and the baseline GABA_A-mediated current noise in female HT relay cells as well as increased sensitivities to THIP and Zn^{2+} . This is consistent with an augmented number of extrasynaptic GABA_ARs containing the δ -subunit, the incorporation of which endows these receptors with unique pharmacological properties (Saxena and Macdonald, 1994; Cope et al., 2005). Biochemical and genetic studies have demonstrated an association between $\alpha 4$ - and δ -subunits in GABA_ARs (Sur et al., 1999; Peng et al., 2002). Together with the raised $\alpha 4$ - and δ -subunit expression levels in the female $\gamma 2^{Y365/367F} +/ -$ thalamus, it strongly suggests that the tonic current was

increased via a genomic effect driving increased expression of $\alpha 4$ - and δ -subunits.

Impact of $\gamma 2^{Y365/367F}$ on dLGN synaptic activity

In addition to increased amplitude, the analysis of synaptic activity revealed an augmented sIPSC frequency in HT relay neurons, usually indicative of increased presynaptic GABA release, which may partly underlie the larger tonic currents observed in these mice. This is consistent with the clear correlation noted between mean sIPSC frequency and amplitude with the tonic current in each female HT dLGN relay neuron.

The increased receptor numbers in HTs may be explained by a developmental impairment within the HT dLGN network. Such an impairment is not surprising since homozygotes for the $\gamma 2^{Y365/367F}$ mutation exhibited a lethal phenotype during embryogenesis, suggesting a key role for GABA_ARs containing the $\gamma 2$ -subunit, and in particular Y365 and Y367, during development (Tretter et al., 2009). The $\gamma 2$ -subunit is expressed early on during embryonic development in rats and humans (Wang et al., 2003; Liu et al., 2005; Kanaumi et al., 2006). Interestingly, $\gamma 2$ -subunit-containing GABA_ARs are important for the development of hindbrain neural networks in the chick embryo (Fortin et al., 1999). GABA_ARs can also participate in the regulation of mammalian neuroblast production and its subsequent differentiation.

Sex-specific neurosteroid modulation of phasic and tonic inhibition in $\gamma 2^{Y365/367F} +/ -$ dLGN

Gender differences in GABA_AR subunit composition and GABA pharmacology have been widely reported (Stock et al., 2000; Gulinello and Smith, 2003; Yee et al., 2004). The loss of THDOC modulation on female HT synaptic activity is significant. THDOC and other neuroactive steroids can directly impact on female GABA_AR subunit expression according to fluctuations in their levels during the estrous cycle (Maguire et al., 2005). Neurosteroids levels can also change following stress, and throughout pregnancy, profoundly altering GABA_AR structure and function (Maguire et al., 2005; Shen et al., 2005); even periods of treatment/exposure to neurosteroids modifies $\alpha 4$ - and δ -subunit levels in female rats (Gulinello et al., 2001; Shen et al., 2005). Furthermore, during changed hormonal states, such as puberty, neurosteroids, which normally increase GABA inhibition, can reduce tonic inhibition via $\alpha 4\beta 2\delta$ GABA_ARs (Shen et al., 2007, 2010). Neurosteroid modulation of GABA_ARs can also be affected by the phosphorylation state of the receptor (and/or its associated proteins) (Fáncsik et al., 2000). This also extends to androgenic steroids whereby the phosphorylation status of murine GABA_ARs, which changes over the estrous cycle, also affects modulation by neurosteroids (Oberlander et al., 2012).

The lack of effect of THDOC in affecting synaptic activity (specifically sIPSC amplitude) in HTs compared with WT could reflect differences in the level of GABA saturation at their respective inhibitory synapses. The inability of THDOC to increase sIPSC amplitudes in HTs would indicate saturation at these synapses and the lack of frequency change by THDOC would further suggest that this effect relies on increased sIPSC amplitude. Thus in HTs, only the kinetic effects of THDOC on rise and decay times are evident, contrasting with WT neurons. The realization that sIPSC amplitude and frequency become insensitive to THDOC in Na_3VO_4 -treated WT neurons possibly reflects the conversion of these synapses from unsaturated to apparently saturated status. Although tyrosine phosphorylation will increase postsynaptic receptor numbers (potentially preventing saturation), ty-

rosine kinases play another important role, since they increase the open probability of GABA channels by ~ 4 -fold (Moss et al., 1995), which would be equivalent to increasing GABA “affinity” and/or efficacy at these synaptic receptors. This change is likely to reduce or ablate the potentiating effect of THDOC on amplitude, producing synaptic saturation, and of note, frequency effects are also abolished, but not the kinetic effects on sIPSC rise and decay times.

Thus, the increased tonic inhibition noted for $\gamma 2^{Y365/367F} +/ -$ female mice may be attributed to a genomic compensatory effect resulting in the increased expression of $\alpha 4$ - and δ -subunits, designed to balance the decreased efficacy of THDOC on inhibitory synaptic activity observed in these animals. Overall, in addition to tyrosine phosphorylation of GABA_AR $\gamma 2$ -subunits modulating inhibitory tone and playing a pivotal role during early neurodevelopment (Tretter et al., 2009), this study reveals a novel and unexpected interaction between GABA_AR-mediated tonic and phasic inhibition following the reduction of neurosteroid sensitivity at inhibitory synapses.

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