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Pharmacological and Biophysical Characteristics of Picrotoxin-Resistant, δ Subunit-Containing GABA_A Receptors

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GABA_A receptors (GABA_ARs) play a crucial role in inhibition in the central nervous system. GABA_ARs containing the δ subunit mediate tonic inhibition, have distinctive pharmacological properties and are associated with disorders of the nervous system. To explore this receptor sub-class, we recently developed mice with δ -containing receptors rendered resistant to the common non-competitive antagonist picrotoxin (PTX). Resistance was achieved with a knock-in point mutation (T269Y; T6'Y) in the mouse genome. Here we characterize pharmacological and biophysical features of GABA_ARs containing the mutated subunit to contextualize results from the KI mice. Recombinant receptors containing δ T6'Y plus WT α 4 and WT β 2 subunits exhibited 3-fold lower EC₅₀ values for GABA but not THIP. GABA EC₅₀ values in native receptors containing the mutated subunit were in the low micromolar range, in contrast with some published results that have suggested nM sensitivity of recombinant receptors. Rectification properties of δ -containing GABA_ARs were similar to γ 2-containing receptors. Receptors containing δ T6'Y had marginally weaker sensitivity to positive allosteric modulators, likely a secondary consequence of differing GABA sensitivity. Overexpression of δ T6'Y in neurons resulted in robust PTX-insensitive IPSCs, suggesting that δ -containing receptors are readily recruited by synaptically released GABA. Overall, our results give context to the use of δ receptors with the T6'Y mutation to explore the roles of δ -containing receptors in inhibition.

Keywords: GABA allosteric modulators, inhibition, antidepressant, dentate gyrus, neurosteroid, ethanol

INTRODUCTION

Activation of GABA_ARs inhibits targets expressing the receptors and sculpts patterns of the activity responsible for thought, emotion, and in fact virtually all brain functions. Two major subclasses of GABA_ARs are prominently expressed in the CNS, sometimes within the same cell type. γ 2-Containing receptors are typically found at synapses and mediate phasic inhibition caused by the

synaptic release of GABA. In contrast, δ -containing receptors are expressed in more circumscribed populations of neurons and tend to mediate tonic currents to ambient GABA and a slow component of IPSCs (Nusser et al., 1998; Wei et al., 2003; Martenson et al., 2017; Sun et al., 2018, 2020). δ -Containing receptors are also thought to have high sensitivity to certain positive allosteric modulators, such as neurosteroids and ethanol (Mihalek et al., 1999; Spigelman et al., 2003; Stell et al., 2003; Wei et al., 2004; Glykys et al., 2007), although our recent results questioned this selectivity (Lu et al., 2020). Features of both GABA_AR subclasses make them attractive targets for therapeutics in different situations. A more nuanced understanding of the properties of δ -containing and γ 2-containing receptors would aid the search for targeted therapeutics and aid understanding of the specific roles of different receptor sub-classes.

To aid the exploration of the two subclasses of GABA_ARs, we recently introduced knock-in (KI) mouse lines that carry pharmacoresistance to picrotoxin (PTX) as a means of isolating the two receptor classes (Sun et al., 2018). Studies of the KI mice revealed a greater contribution of δ -containing receptors to IPSCs of dentate granule cells (DGCs) than expected (Sun et al., 2018, 2020). The KI mice also revealed little or no preferential sensitivity to neurosteroids (Lu et al., 2020). An open question is whether receptors containing the mutant δ subunit are sufficiently different pharmacologically from WT δ -containing receptors to explain the unexpected results.

PTX-resistant GABA_AR subunits were engineered and partially characterized previously. It was found that the T6'Y mutation could be made in any subunit of a pentameric GABA_AR to reduce or eliminate PTX sensitivity (Gurley et al., 1995; Sedelnikova et al., 2006; Erkkila et al., 2008). In γ 2 receptors, this amino acid substitution had little effect on GABA EC₅₀ (Erkkila et al., 2008), but when introduced into mice, γ 2T6'Y causes a seizure phenotype that appears to arise from slightly altered kinetics of synaptic currents (Sun et al., 2019). The comparable mutation in δ subunit-containing receptors has not been characterized in as much detail, although we have shown that kinetics and agonist sensitivity are similar to WT in native DGCs (Sun et al., 2018, 2019).

Here we use a combination of recombinant receptor subunits, where we can directly compare WT and mutated subunits, and tissue slices, where subunits are expressed in a native environment, to examine pharmacological and biophysical properties of GABA_ARs that may be important to their physiological role. We found that receptors with a mutated δ subunit exhibit ~3-fold higher sensitivity to GABA, leading to marginally altered responsiveness to allosteric modulators. We also found that mutant receptors exhibit similar rectification to WT δ -containing receptors and γ 2-containing receptors in native cells. Thus, rectification is unlikely to increase the prominence of δ -containing receptors relative to γ 2-containing receptors at transmembrane voltages positive to rest. Overall, our results show that pharmacoresistant KI mice are valid tools for evaluating the roles of GABA_AR sub-classes if caveats are considered.

METHODS

Cell Culture and Recombinant Receptor Expression

The murine neuroblastoma Neuro-2a (N2a; ATCC No. CCL-131) cell line was grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% (v/v) FBS, 2 mM glutamine plus 100 U/ml penicillin, and 0.1 mg/ml streptomycin in an atmosphere of 5% CO₂ and 95% air, and was maintained at sub-confluent densities in the growth media.

GABA_ARs were expressed in N2a cells by transiently transfecting the cDNAs of free α 4, β 2, and δ subunits of the GABA_AR. The cDNA for human α 4 was obtained from Dr. Paul Whiting (Merck, Harlow, Essex, UK), rat β 2 was obtained from Dr. David Weiss (University of Texas Health Science Center, San Antonio, TX, USA), and rat δ was provided by Dr. Robert Macdonald (Vanderbilt University, Nashville, TN, USA). The δ (T269Y; δ T6'Y) mutation was used to confer resistance to PTX (Gurley et al., 1995). The mutations were made using the QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). The δ subunit contained the FLAG epitope in the amino terminus of the subunit (Ueno et al., 1996). Transfection was carried out using a total of 1.2 μ g of cDNA in the ratio of 1:1:2(α : β : δ), along with reporter plasmid (GFP, Clontech Laboratories, Mountain View, CA) as a positive transfection marker. Lipofectamine2000 (Life Technologies, Carlsbad, CA) was used as a transfection reagent according to the manufacturer's protocol. Electrophysiological experiments were performed 48–72 h later following transfection.

Rat primary cultures of hippocampal cells were prepared from 1 to 3 day postnatal Sprague Dawley rats, as described previously (Mennerick et al., 1995). Tissue was prepared according to protocols approved by the Institutional Animal Care and Use Committee. The GABA_AR subunits were transduced with AAV8-Syn- α 4-IRES-GFP, AAV8-Syn- δ T6'Y-IRES-GFP, and AAV8-Syn-GFP virus 5 days after plating (Syn refers to the human synapsin promoter). Viral preparation was supported by the Hope Center Viral Vectors Core at Washington University School of Medicine. Electrophysiological experiments were performed 7 days following viral infection.

Whole-Cell Patch-Clamp Recording in Cell Cultures

Experiments on N2a cells were conducted using standard whole-cell techniques. Non-transfected cells had no response to GABA. The bath solution containing (in mM): 138 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose, and 10 HEPES; pH 7.25. Patch pipettes were filled with an internal solution containing (in mM): 130 CsCl, 4 NaCl, 4 MgCl₂, 0.5 CaCl₂, 5 EGTA, 10 HEPES, pH 7.25. When filled with this solution, pipette tip resistance was 3–6 M Ω . Cells were clamped at -70 mV. Drugs and elevated potassium were applied with a multibarrel, gravity-driven local perfusion system. The estimated solution exchange times were 10–20 ms, measured by the change in junction currents at the tip of an open patch pipette. A typical drug application consisted of recording 2 s of baseline, followed by a 4–8 s drug application

and a bath application (up to 1 min) until full recovery. Culture recordings were performed at room temperature.

Currents were filtered at 2 kHz and recorded at 5 kHz with an Axopatch 200 B amplifier (Molecular Devices, San Jose, CA). The analysis of current traces aimed at determining the peak current was conducted using pClamp 9.0 software (Molecular Devices). All recordings were performed at room temperature. Charge calculations for phasic currents were performed in Clampfit with cursors set at the onset and of K⁺ application and using the built-in statistics functions of Clampfit to calculate the integral of current over time.

EC₅₀ values for GABA and THIP were derived from fits to the Hill equation with the Hill coefficient constrained to ≤3 for both GABA and THIP to account for multiple binding sites. Comparisons of the sensitivity to agonist were made by the Extra sum-of-squares F test implemented in GraphPad Prism software with a p criterion of 0.05. Alternatively, individual cells were fit by the Hill equation, followed by a one-way ANOVA and Sidak's multiple comparisons test if the ANOVA revealed a difference. For the latter approach, THIP data from 4 WT cells and 5 cells in the δT6'Y conditions were excluded due to non-converging fits or outlier EC₅₀ values (>5 standard deviations beyond mean).

Slice Preparation

Mice from WT, δT6'Y KI, and γ2T6'Y KI of either sex were used for experiments at postnatal day (P) 30 ± 2 days (Sun et al., 2018). Coronal brain slices at 300-μm thickness were cut in ice-cold, modified artificial CSF (aCSF containing in mM: 87 NaCl, 75 sucrose, 25 glucose, 25 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, equilibrated with 95% oxygen-5% CO₂ plus 0.5 CaCl₂, 3 MgCl₂; 320 mOsm). Slices were incubated in choline-based recovery aCSF (in mM: 92 choline chloride, 25 glucose, 30 NaHCO₃, 2.5 KCl, 1.2 NaH₂PO₄, 20 HEPES, 2 thiourea, 5 Na ascorbate, 3 Na pyruvate, 2 CaCl₂, and 1 MgCl₂, oxygenated; 300 mOsm) at 32°C for 30 min, and then stored in regular aCSF (in mM: 125 NaCl, 25 glucose, 25 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, oxygenated; 310 mOsm) for at least 1 h at 25°C before experimental recording. To measure GABA_AR activation, 10 μM NBQX and 50 μM D-APV were added in the regular aCSF to inhibit ionotropic glutamate receptors.

Slice Whole-Cell Recording

Brain slices were transferred into a recording chamber with oxygenated, regular aCSF perfused at 2 ml/min at 32°C. Hippocampal DGCs were identified by IR-DIC microscopy (Nikon FN1 microscope and Photometrics Prime camera). Whole-cell recordings were performed with pipettes pulled from borosilicate glass and with open tip resistance of 3–7 MΩ. Pipettes contained the following in mM: 130 CsCl, 10 HEPES, 5 EGTA, 2 MgATP, 0.5 NAGTP, and 4 QX-314; pH adjusted to 7.3 with CsOH; 290 (mOsm). Recordings began 5 min after whole-cell configuration was established. Signals were recorded using a MultiClamp 700B amplifier (Molecular Devices), Digidata 1550 16-bit A/D converter, and pClamp 10.4 software (Molecular Devices). To measure (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol) THIP currents, DGCs were voltage-clamped at –70 mV, and PTX (50 μM) was applied

to block non δ-containing receptors in δT6'Y CRISPR KI slices, and the holding current was recorded throughout.

To measure the GABA sensitivity of δ-containing receptors, nucleated patches were obtained (Sather et al., 1992) to minimize the influence of GABA uptake on GABA responses. After PTX application, GABA at 1, 10, and 30 μM was applied. Data were plotted in Prism (Graphpad, San Diego, CA).

To measure subunit contributions to tonic current and to measure rectification of GABA_AR-mediated current, DGCs from WT, δT6'Y KI, or γ2T6'Y KI slices were recorded at –70 mV. Exogenous GABA (5 μM) was applied to induce a basal tonic current, followed by PTX co-application (50 μM) to separate δ-containing receptors from γ2-containing receptors in the two KI lines. The competitive GABA_AR antagonist gabazine (GBZ, 50 μM) was applied to block the remaining GABA_ARs at the end of recordings. Current remaining after the GBZ application was taken as a 0-level tonic current. For rectification, the current-voltage (*I*-*V*) relationship of tonic currents mediated by δ-containing receptors or γ2-containing receptors was measured by applying a slow (10 s) voltage ramp from –100 mV to 60 mV in the presence of Cd²⁺ to block voltage-gated Ca²⁺ channels. Rectification index (RI) represents the ratio of conductance of GABA_ARs at –60 mV and +60 mV and was calculated as follows: $RI = [I_{+60}/(60 - E_{rev})]/[I_{-60}/(-60 - E_{rev})]$.

Drugs

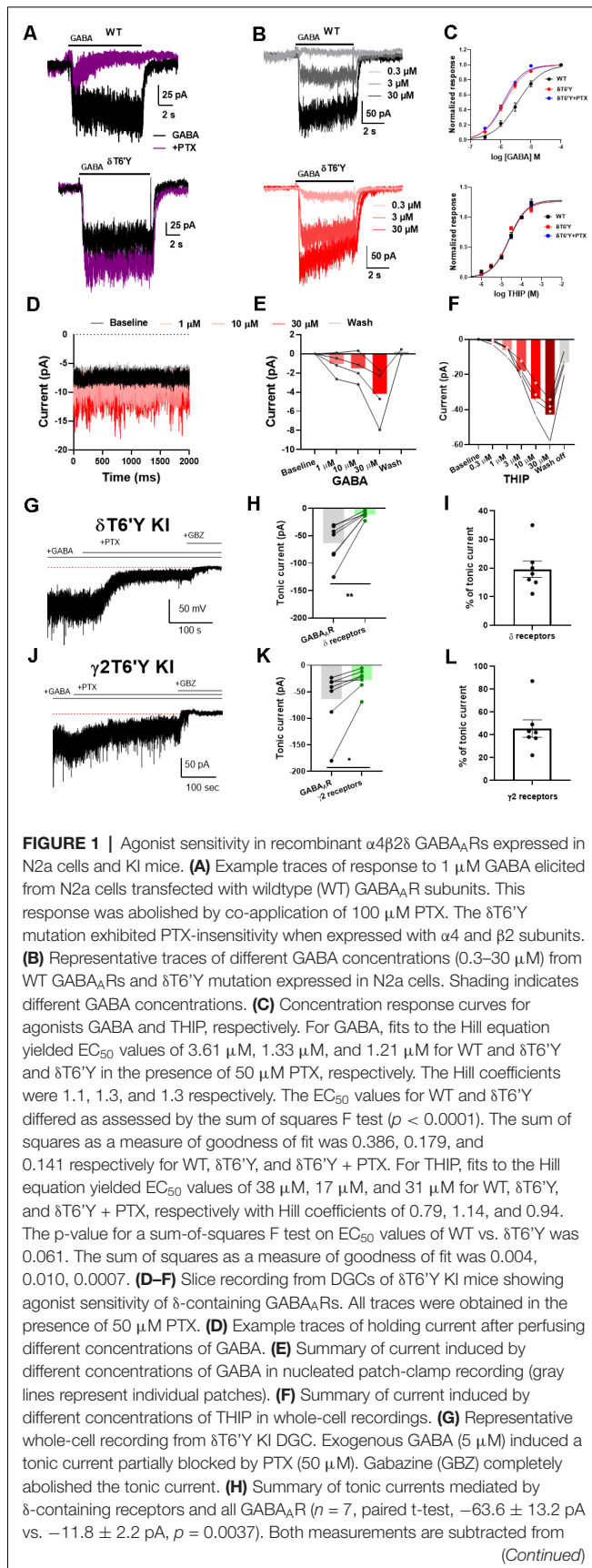
GABA, pentobarbital, and salts were purchased from Sigma-Aldrich (St. Louis, MO). DS2 [4-chloro-N-2-(2-thienyl)imidazo[1,2-a]pyridine-3-yl benzamide] was purchased from R&D Systems (Minneapolis, MN). Steroids were from Sigma-Aldrich, or Steraloids (Newport, RI). Channel blockers NBQX and D-APV were from Tocris Bioscience (Minneapolis, MN).

RESULTS

Agonist Sensitivity in Recombinant GABA_ARs and CRISPR Mutation Knock-In Mice

To characterize pharmacological and biophysical features of GABA_ARs containing the δT6'Y subunit, we first examined activation by the natural agonist GABA. **Figure 1A** shows a typical response elicited by 1 μM of GABA from wild type (WT) α4β2δ subunits transfected into N2a cells. The response was strongly inhibited when co-applied with the channel blocking antagonist PTX at 100 μM. PTX sensitivity was absent when δT6'Y was introduced with WT α4 and β2 subunits, but the activation by GABA was retained. Overall, there was no difference in the amplitude of GABA responses to 1 μM GABA in the presence or absence of 50 μM PTX (–129.7 ± 21.5 pA vs. –138.4 ± 22.2 pA, *n* = 27, 26; *p* = 0.78, *t*-test). We note that incorporation of δ subunits into recombinant receptors has been controversial, and PTX insensitivity of δT6'Y (or the comparable mutation in other subunits) yields a useful marker of subunit incorporation.

To further understand the impact of this mutation on the function of GABA_ARs, we investigated a full concentration-

**FIGURE 1 |** Continued

the residual holding current in the presence of GBZ. **(I)** Percentage of tonic current mediated by δ -containing receptors ($n = 7$, $19.6\% \pm 2.9\%$). **(J–L)** The same experiment from $\gamma 2\delta$ Y KI DGCs. Similar to δ T6'Y KI DGCs, PTX partially blocked the GABA-induced tonic currents ($n = 7$, paired t-test, -63.2 ± 21.0 vs. -27.2 ± 7.9 pA, $p = 0.0383$). The contribution of $\gamma 2$ -containing receptors was calculated ($n = 7$, $45.4\% \pm 7.6$), with the balance ($\sim 55\%$) mediated by non- $\gamma 2$ containing receptors. PTX, picrotoxin; KI, knock-in; DGCs, dentate granule cells.

response relationship by varying GABA concentration in WT and δ T6'Y receptors (**Figures 1B,C**). We observed that the EC₅₀ of receptors containing the δ T6'Y mutation was left-shifted when compared to WT δ -containing receptors (1.33 μ M and 3.61 μ M, respectively, $p < 0.001$, Extra Sum of Squares F test). Alternatively, comparison of EC₅₀ values by fits to data from individual cells by one-way ANOVA (see “Methods” section) showed similar results: overall effect on EC₅₀ ($F_{(2,21)} = 21.77$; $p < 0.001$), with *post hoc* comparison revealing a difference between genotypes (adjusted p -value < 0.0001) but not between PTX conditions in the δ T6'Y cells. However, we failed to detect a lower EC₅₀ for the δ -preferring agonist THIP (**Figure 1C** lower panel 38.7 μ M and 16.7 μ M for WT and δ T6'Y mutated group, respectively; $p = 0.06$, Extra Sum of Squares F test). Individual fits for THIP revealed a similar result: (one-way ANOVA $F_{(2,29)} = 2.596$, $p = 0.092$)

In addition, we performed GABA and THIP concentration-response experiments on receptors bearing the δ T6'Y mutation in the presence of 50 μ M PTX (**Figure 1C**, blue symbols). The EC₅₀ for GABA but not THIP was detectably altered by the δ T6'Y mutation, and PTX presence did not alter the EC₅₀ on the mutated receptors (**Figure 1**). These values were very similar to those in the absence of PTX, further highlighting the lack of significant PTX effect on mutant receptor function.

The finding of reduced agonist EC₅₀ values in recombinant GABA_ARs for the natural agonist GABA leads to the question of how agonists behave on δ GABA_ARs in native cells, especially since WT recombinant δ -containing receptors exhibit a range of estimated EC₅₀ values, from low nM to high μ M (Brown et al., 2002; Karim et al., 2012, 2013; Eaton et al., 2014; Wongsamitkul et al., 2016). Although we could not evaluate WT native δ GABA_ARs because of the lack of selective pharmacology, we evaluated δ -containing GABA_ARs using PTX-resistant δ T6'Y KI DGCs, a cell type rich in δ -containing receptors. We performed nucleated patch recordings, lifted from the slice, to avoid the complication of GABA uptake, which lowers the local GABA concentration in tissue to indeterminate values (Isaacson et al., 1993). In the presence of PTX, exogenous GABA increased current in nucleated patches from 1 μ M to 30 μ M and recovered after GABA wash off (**Figures 1D,E**). The results suggest that the increased sensitivity to GABA evident in recombinant GABA_ARs is not readily evident in native cells. To examine receptor sensitivity using a method that doesn't suffer from the disadvantages of patch excision and for which recombinant receptors did not exhibit clear alteration in sensitivity, we examined whole-cell current activated by the poorly transported δ -preferring agonist THIP. Similar to GABA application, the

current mediated by δ -containing receptors increased as THIP concentration increased from 1 μM to 30 μM (**Figure 1F**). These results show that in DGCs the EC_{50} for both natural and δ -preferring agonists is in the micromolar range rather than in the nM range as has been observed in some recombinant receptor experiments.

Given that the response of $\delta\text{T6}'\text{Y}$ -containing receptors in native cells does not appear to exhibit the higher sensitivity to GABA observed in recombinant receptors, we evaluated the contribution of δ -containing receptors to GABA-induced tonic current using both $\delta\text{T6}'\text{Y}$ and $\gamma\text{2T6}'\text{Y}$ knock-in mouse lines (Sun et al., 2018; Lu et al., 2020). In mouse hippocampal DGCs, we induced tonic current with 5 μM GABA, typical of previous work (Lee and Maguire, 2014). In both $\delta\text{T6}'\text{Y}$ KI and $\gamma\text{2T6}'\text{Y}$ KI DGCs, 5 μM GABA induced ~ 60 pA tonic current. This current was partially blocked by 50 μM PTX, leaving PTX-resistant tonic current. GBZ (50 μM) was applied to eliminate the remaining tonic current (**Figures 1G,J**). Surprisingly, δ -containing receptors mediated $\sim 20\%$ of the GABA-induced tonic current (**Figures 1H,I**), judged by PTX-resistant current in $\delta\text{T6}'\text{Y}$ KI cells. By contrast, non- γ2 containing receptors mediated 55% of the tonic current from the $\gamma\text{2T6}'\text{Y}$ cells (**Figures 1K,I**). Both mouse lines indicate a smaller contribution of δ -containing receptors to GABA-induced tonic current than previous work has suggested. Possible reasons for the discrepancy in estimates for δ contribution within the two KI mouse lines are evaluated in the Discussion.

Rectification Does Not Differ Among Native GABA_AR Isoforms

We extended the examination of native receptors in DGCs to rectification properties. GABA_AR-mediated tonic current shows strong outward rectification in CA1 pyramidal cells (Pavlov et al., 2009). Outward rectification of conductance in the range of voltages from -70 mV to near action potential threshold will influence excitability, and disproportionate outward rectification of GABA current mediated by δ -containing receptors could perhaps help explain the small contribution of these receptors in our experiments compared with previous work (Stell et al., 2003; Wei et al., 2003). To examine whether outward rectification is stronger in δ -containing GABA_ARs than in γ2 -containing receptors, we measured the tonic current of DGCs while applying a slow voltage ramp from -100 mV to $+60$ mV (**Figures 2A–E**). After applying sequential PTX and GBZ in slices from PTX-resistant $\delta\text{T6}'\text{Y}$ KI and $\gamma\text{2T6}'\text{Y}$ KI mice, we calculated the rectification index of GABA current mediated by δ -containing receptors and by γ2 -containing receptors, respectively (**Figures 2A–E**). Our results suggest that rectification is similar among the three genotypes (**Figures 2B,D,F**).

Positive Allosteric Modulator (PAM) Sensitivity in Recombinant Receptors

Many important experimental and clinically used compounds augment GABA_AR function. Do these compounds act as expected at receptors containing the $\delta\text{T6}'\text{Y}$ mutation? We tested pentobarbital, a barbiturate; allopregnanolone (AlloP), an endogenous neurosteroid; DS2, a δ -receptor preferring positive

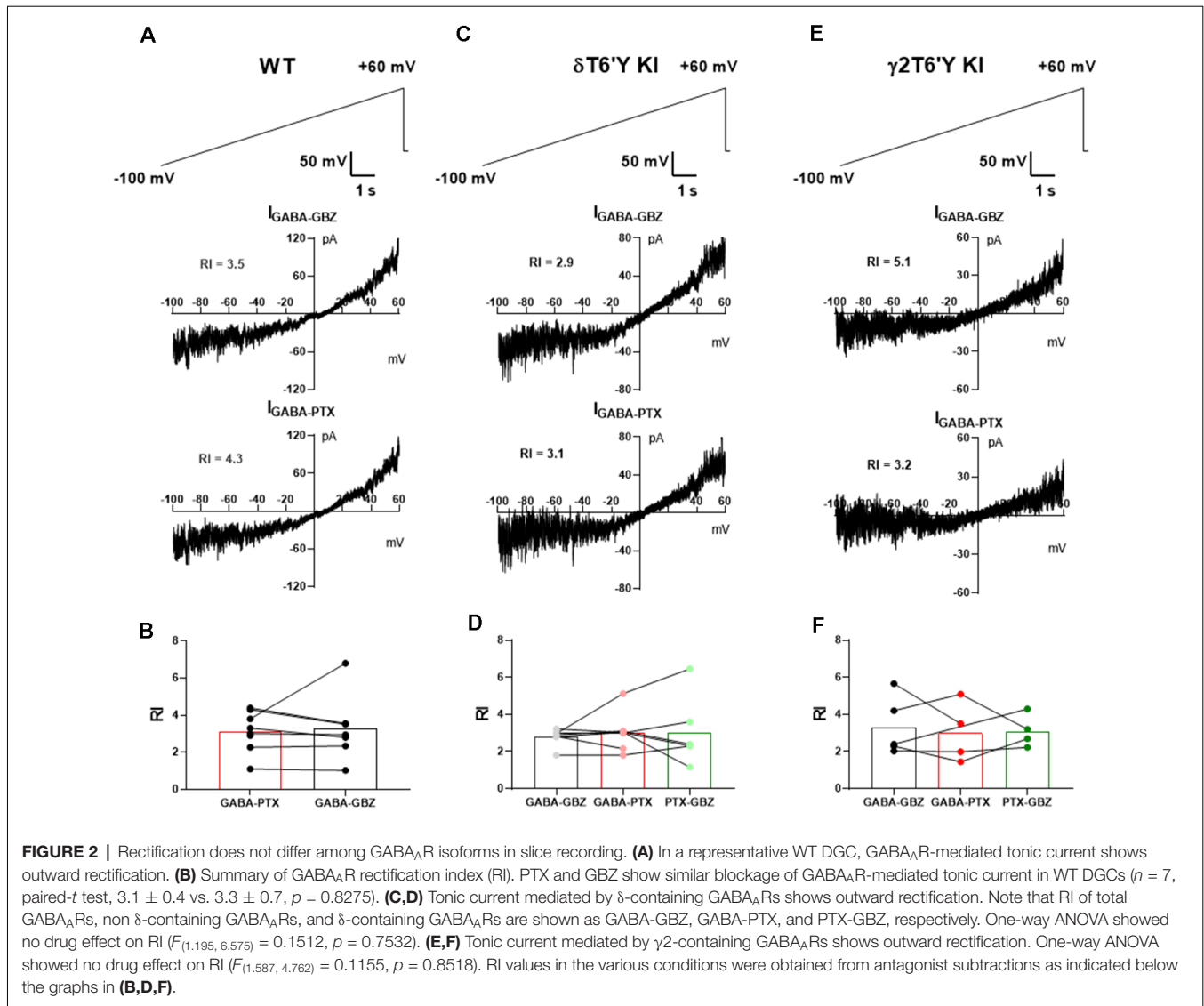
allosteric modulator (PAM; Jensen et al., 2013); and ethanol. **Figure 3** shows that pentobarbital (50 μM), AlloP (50 nM), and DS2 (1 μM) all potentiated responses to 1 μM GABA in both WT and $\delta\text{T6}'\text{Y}$ receptors (**Figures 3A,B**). A two-way ANOVA applied to data in **Figure 2B** revealed that overall, $\delta\text{T6}'\text{Y}$ exhibited less potentiation from modulators ($F_{(2, 145)} = 10.04$, $p < 0.001$). However, *post hoc* comparisons showed only marginal differences for each modulator (**Figure 3B**). Ethanol modestly potentiated both WT and $\delta\text{T6}'\text{Y}$ currents at 30–100 mM (**Figure 3C**; 100 mM shown). We attribute the overall reduced sensitivity of $\delta\text{T6}'\text{Y}$ receptors to positive modulators to be the likely secondary consequence of reduced agonist EC_{50} (**Figure 1**). Overall, we conclude that positive allosteric modulators act similarly in WT and $\delta\text{T6}'\text{Y}$ -containing receptors.

DS2-Sensitive PTX-Insensitive Responses in Rat Hippocampal Cultures

Hippocampal neurons prominently express γ2 -containing GABA_ARs that traffic mainly to synapses, while δ -containing receptors are located perisynaptically or extrasynaptically, mainly in DGCs in the hippocampus (Wei et al., 2003). Because δ -containing receptors are thought to mediate mainly tonic GABA currents, we tested whether δ receptors are excluded from activation by synaptic GABA release. We used viral transduction of $\delta\text{T6}'\text{Y}$ along with the preferred δ partner α4 in hippocampal neurons. GFP + α4 and non-transduced cells were used as controls. First, we evaluated the functional expression of transduced subunits. We used PTX resistance and sensitivity to the δ -preferring PAM DS2 to implicate δ -containing receptors. **Figures 4A,B** shows representative responses to 0.2 μM GABA \pm 1 μM DS2 in the presence of PTX in control and $\delta\text{T6}'\text{Y}$ neurons. WT cells demonstrated that there was little “breakthrough” GABA current in the presence of PTX. These examples and summary data in **Figure 4C** in the absence of PTX suggest that control cultures have almost no endogenous δ in functional receptors, probably owing to the prominence of pyramidal neurons rather than granule neurons in the cultures. $\delta\text{T6}'\text{Y}$ alone (without α4) was also capable of sustaining responses to GABA and DS2, suggesting that $\delta\text{T6}'\text{Y}$ can partner with endogenous α and β subunits to form functional receptors (**Figure 4C**).

Synaptic Activation of Overexpression of $\delta\text{T6}'\text{Y}$ Mutation in Rat Hippocampal Cultures

Given the robust expression of $\delta\text{T6}'\text{Y}$ in hippocampal cultures (**Figure 4**), we explored whether δ receptors are accessed by the synaptic release of GABA. We recorded from infected cells while challenging the surrounding network with 6 mM potassium in the presence of PTX and the glutamate receptor blockers NBQX and D-APV. The currents in **Figure 5A** thus represent PTX-insensitive IPSCs from the recorded cell. The contribution of δ receptors was verified with DS2 (1 μM) application, which prolonged IPSCs (**Figures 5A,E**). IPSCs were also sensitive to GBZ, a competitive GABA_AR antagonist (**Figure 5A3**). In control GFP-only cells (also recorded in the presence of receptor antagonists), potassium-elicited currents were much smaller



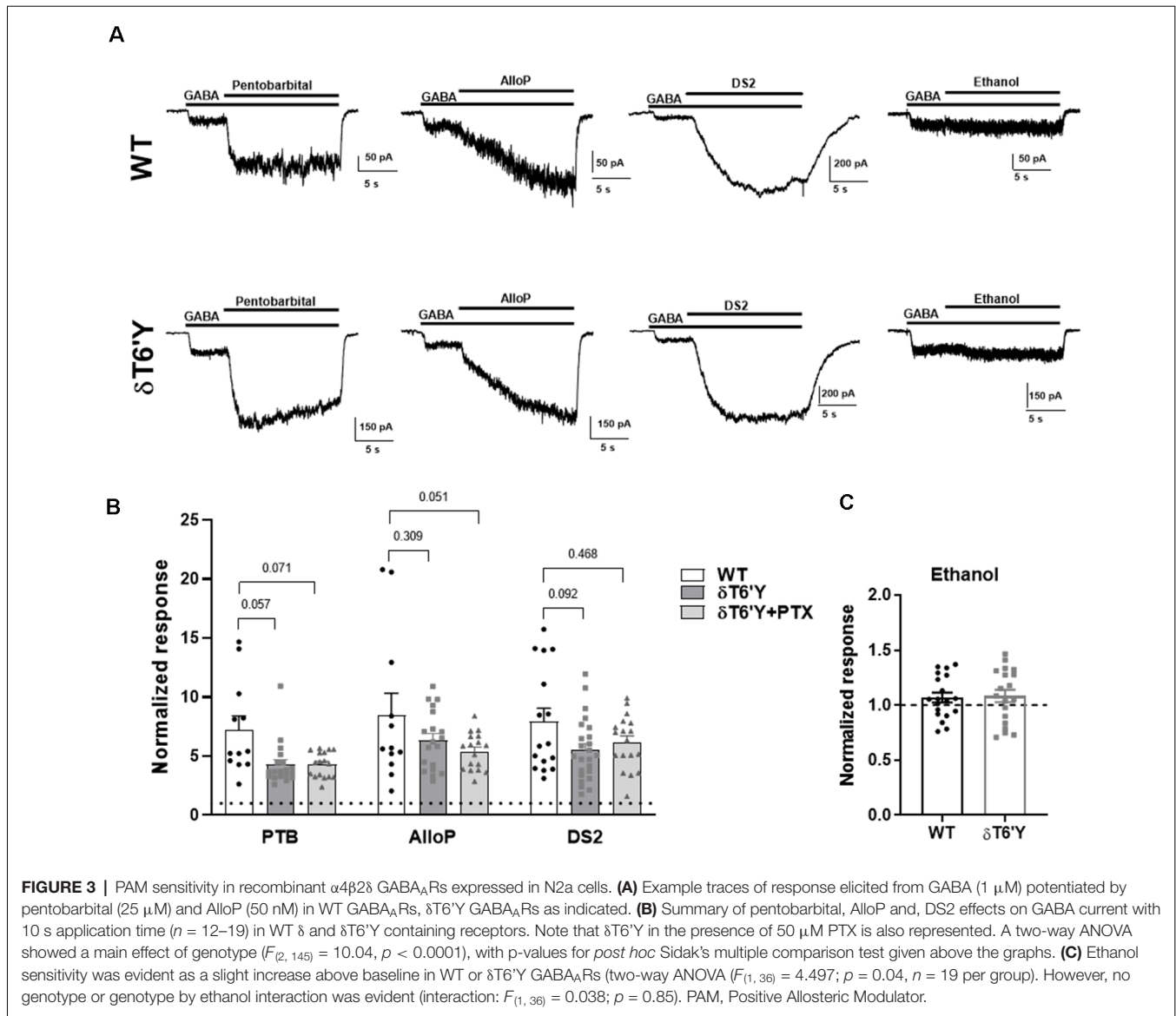
(Figures 5B,D), reflecting primarily direct potassium effects, and were insensitive to DS2 and to GBZ (Figures 5B1–B3,E). To test that phasic events were indeed synaptically driven, we confirmed that lowering bath Ca^{2+} eliminated the phasic events in each of the 10 cells tested (Figure 5C). We conclude that δ subunit-containing receptors are readily activated by synaptically released GABA, at least under conditions of exogenous transduction. Note that our experiments do not determine whether the activated receptors are anatomically synaptic, perisynaptic, or extrasynaptic.

DISCUSSION

We have evaluated the effect on the receptor function of a point mutation in the δ subunit of GABA_ARs expressed heterologously and as a knock-in in DGCs. The point mutation has the intended effect on PTX sensitivity. However, the results also show that the mutation is not completely silent in the absence of PTX.

The primary change in receptor function is a reduction in GABA EC_{50} in recombinant GABA_ARs, which may also account for the reduced sensitivity of receptors to positive allosteric modulators at a fixed agonist concentration. EC_{50} differences could arise from genuine changes in the binding properties of the receptor or from changes to the gating properties of the receptor (Colquhoun, 1998). Given that the amino acid substitution occurs within TM2, near important pore-forming residues, it seems likely that gating properties are primarily affected and agonist binding affected indirectly.

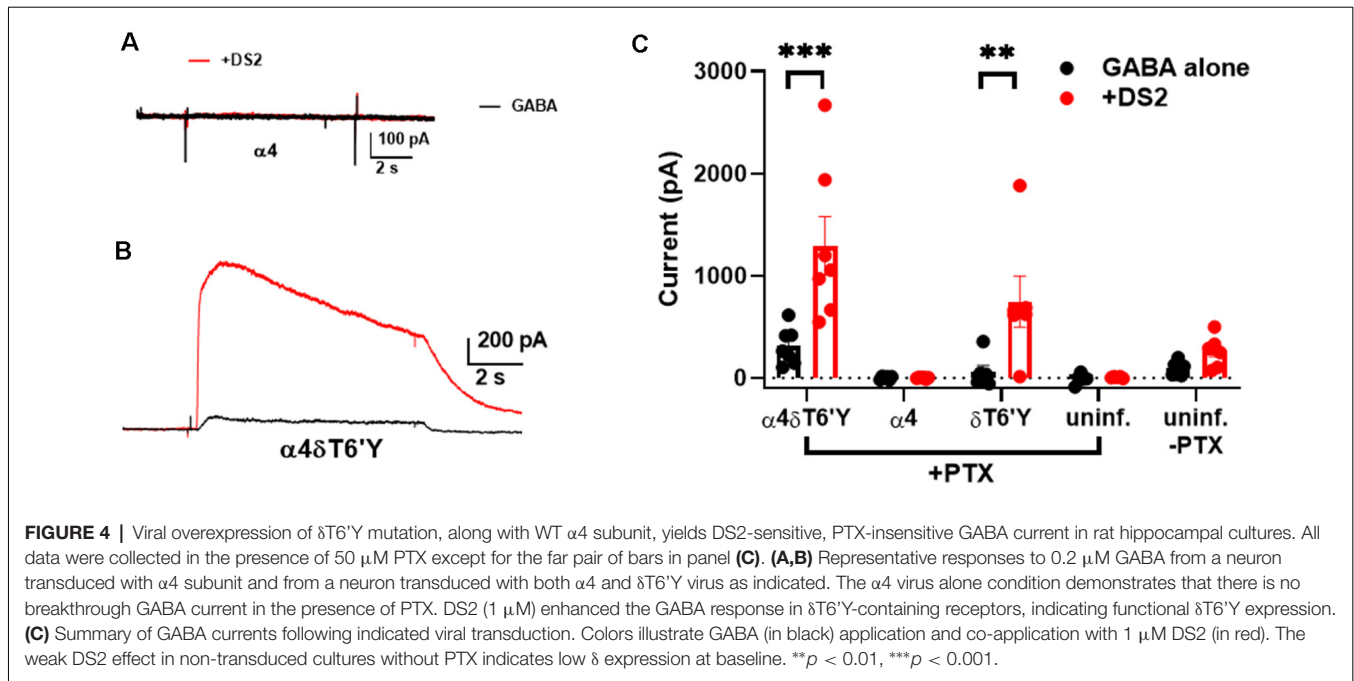
A puzzling attribute of the mutant receptors is the altered EC_{50} for GABA but not for THIP (Figure 1). Although speculative, a possible reason for the difference is the partial agonist quality of GABA compared with the full agonist properties of THIP at δ subunit-containing receptors (Brown et al., 2002). The mutation may affect the EC_{50} for partial agonists but not for full agonists, because of different receptor conformations adopted by bound full vs. partial agonists.



We previously used δ T6'Y mice, also employed in the current study, along with γ 2T6'Y knock-in mice to argue that δ receptors contribute more to synaptic IPSCs in DGCs than prevailing views typically suggest (Sun et al., 2018, 2020). Our present experiments reinforce these observations with an acute expression of δ subunits in primary cultures (Figures 4, 5). Several pieces of evidence suggest that the phasic events are of synaptic (vesicular) origin rather than from reverse GABA uptake. First, the events are Ca^{2+} dependent (Figure 5C). Although interactions between synaptic glutamate release and reverse GABA transport are formally possible (Guimarães-Souza et al., 2011), reverse GABA transport is evoked by stronger depolarization and with a slower time course than in our experiments (Gaspary et al., 1998; Allen et al., 2004). Note that neither our previous experiments nor the present experiments speak to the anatomical location of the δ containing receptors. Rather, the results suggest that δ receptors can be accessed by synaptic GABA release and contradict the

view that δ receptors solely mediate tonic current. Previously, using PTX-resistant γ 2 knock-ins, we used PTX isolation and digital subtraction to isolate WT δ IPSCs in DGCs and failed to discern a difference in amplitude or decay kinetics of δ mediated IPSCs, between WT and δ T6'Y IPSCs (Sun et al., 2018, 2020), based on the assumption that δ receptors in γ 2T6'Y mice retain WT features. Therefore, it appears that the mutation has a limited functional impact on DGCs. The possible reasons are considered below.

The lower EC_{50} to GABA of δ T6'Y receptors predicts a larger tonic current in T6'Y DGCs in response to a fixed, low GABA concentration, but this prediction was not borne out. We also previously showed that responses to a sub-saturating concentration of the δ -preferring agonist THIP were not discernibly different from those of WT receptors in DGCs (Sun et al., 2018; Lu et al., 2020). If tonic GABA responses were augmented because of the lower agonist EC_{50} in T6'Y receptors,

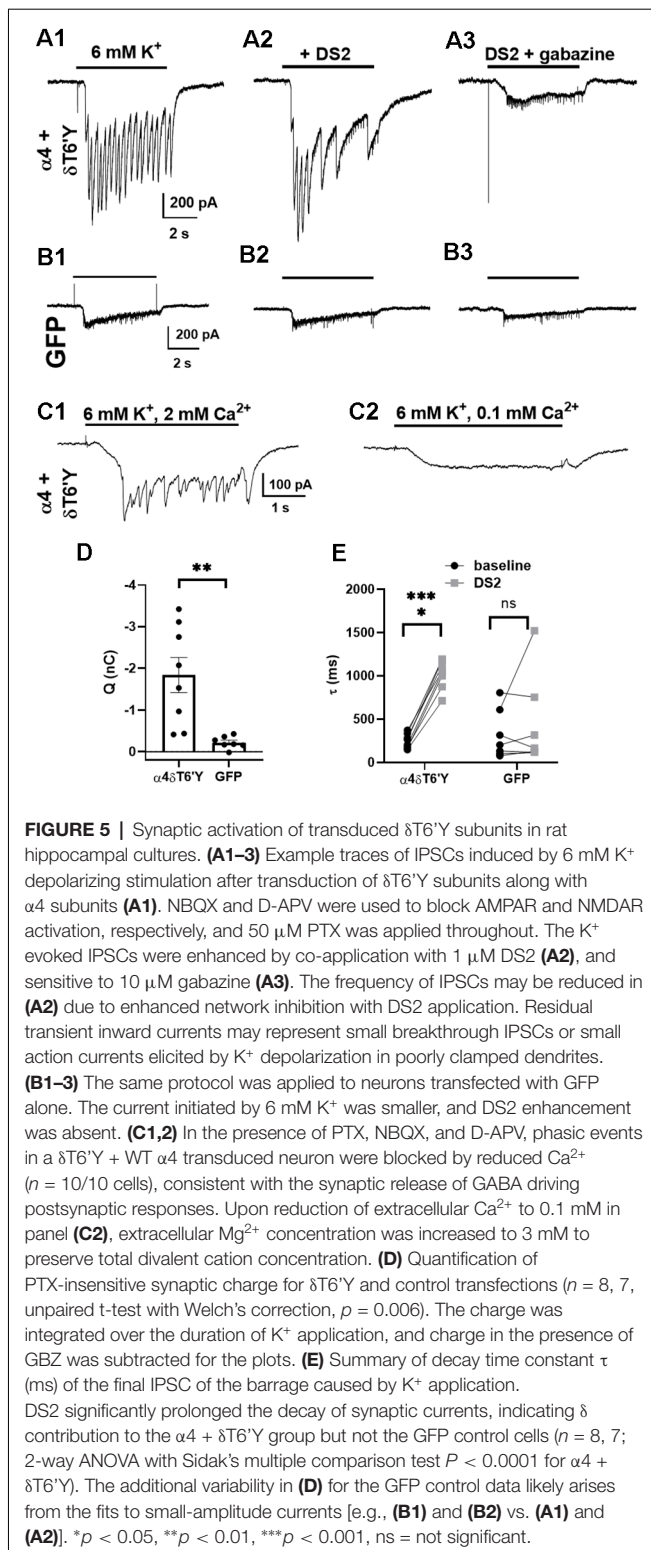


we may have overestimated δ contribution, exacerbating the contrast with prevailing views. Here we also examined a range of agonist concentrations in $\delta T6'Y$ KI cells to test a hypothesis of exceptionally high-affinity delta receptors (Karim et al., 2012; Eaton et al., 2014). Our study constitutes the first direct assessment of GABA sensitivity of δ receptors in native cells and suggests that the EC_{50} of DGC δ receptors is in the micromolar range, even with a possible three-fold lowering induced by mutation. Thus, at least in DGCs, GABA and THIP are not particularly high-potency agonists at native δ receptors. Although the EC_{50} values differ from some published values (Karim et al., 2012; Eaton et al., 2014; Pan et al., 2018), our observations are in line with others (You and Dunn, 2007; Mortensen et al., 2010). Our results in fact appear consistent with a particular subunit arrangement in oocytes, forced by concatemeric subunits (Eaton et al., 2014).

Our results show that δ -containing receptors mediate $\sim 20\%$ of $GABA_A$ R tonic current in $\delta T6'Y$ KI DGCs, while in $\gamma 2 T6'Y$ KI DGCs, non $\gamma 2$ -containing receptors mediate $\sim 55\%$ of the tonic current. Although both are lower than previous estimates, a possible reason for the internal discrepancy is that non- $\gamma 2$ receptors besides δ receptors (e.g., $\gamma 1$ -containing receptors and $\alpha\beta$ -containing receptors) contribute to PTX-sensitive current in $\gamma 2 T6'Y$ cells (Mortensen and Smart, 2006). The lower estimate of contribution from δ receptors in $\delta T6'Y$ cells suggests that high agonist affinity of $\delta T6'Y$ (Figure 1C) does not dominate, consistent with direct studies of GABA EC_{50} in DGCs in Figures 1D,E. Poor functional expression of $\delta T6'Y$ receptors might explain the low δ contribution to GABA tonic current in $\delta T6'Y$ cells, but previously we presented multiple lines of evidence that $\delta T6'Y$ expression is near that of WT in DGCs (Sun et al., 2018). Further, in our current work the total GBZ-sensitive GABA current amplitude in the two mouse lines did not differ

(Figures 1H,K). Thus, the evidence seems to favor non- $\gamma 2$ /non- δ receptor contributions to the tonic current to explain the discrepancy in Figure 1I vs. Figure 1L.

Positive allosteric modulator sensitivity is important since many drugs of this class are in clinical use or in clinical development. Subunit selective modulators could represent a new generation of therapeutics (Rudolph and Möhler, 2006, 2014). For instance, neurosteroids may have persistent antidepressant effects by virtue of selectivity for δ receptors (Maguire and Mody, 2008; Melón et al., 2018; Meltzer-Brody and Kanen, 2020). On the other hand, our recent studies suggested that δ receptors in the presence or absence of GABA or THIP seem to exhibit little if any neurosteroid selectivity over $\gamma 2$ receptors (Lu et al., 2020). Here we found that recombinant $\delta T6'Y$ receptors retained sensitivity to allosteric modulators, including AlloP and ethanol, with both WT and $\delta T6'Y$ receptors exhibiting very limited sensitivity to ethanol in our hands. This finding differs from some previous results (Wallner et al., 2006) but not others (Borghese et al., 2006). Although an analysis of variance showed an effect of the $\delta T6'Y$ mutation on positive allosteric modulation, *post hoc* analyses of individual PAMs indicated this global effect was largely accounted for by reduced efficacy of PTB (Figure 3). Because very different classes of compounds were used in these experiments and no one compound accounted entirely for the reduced overall effect (Figure 3), we hypothesize that the change in agonist sensitivity ultimately underlies the difference in the effects of modulators at a fixed agonist concentration. The increased effectiveness of a fixed agonist concentration at mutated receptors yields a smaller dynamic range for PAMs. In our previous studies, this effect could have caused us to underestimate AlloP potentiation in native cells bearing the $\delta T6'Y$ mutation. However, complementary results in native cells



bearing $\gamma 2T6Y$ suggest that our conclusions in native cells about AlloP selectivity are not affected by this property of $\delta T6Y$ receptors (Lu et al., 2020). In addition, results in **Figure 1E** suggest that GABA sensitivity in native cells may not be altered as they are in recombinant receptors (**Figure 1C**).

There are several reasons that differences in agonist sensitivity observed in recombinant receptors might not be evident in DGC native receptors. We cannot completely exclude the possibility that native cells exhibit the same three-fold higher sensitivity for GABA observed in recombinant receptors; experiments in native cells embedded in tissue slices may lack the precision obtainable in cultures, where exposure to solution and drug is highly controlled. On the other hand, there are potential reasons that a change observed in recombinant receptors expressed in heterologous cells may not be evident in native receptors. One possibility is that the beta subunit differs in our recombinant receptors vs. native cells, which could alter channel behavior. Another possibility is that post-translational modification or interacting proteins differ in the two environments. Regardless, the caveat of agonist potency will be important to bear in mind when studying other cell types.

GABA_ARs have voltage sensitivity that has been previously suggested to contribute to inhibition by tonic current (Weiss, 1988; Pavlov et al., 2009). However, to our knowledge rectification has been examined in recombinant δ GABA_ARs (Brown et al., 2002) but not native δ -containing GABA_ARs. We used $\delta T6Y$ to explore the hypothesis that δ receptors may exhibit more rectification than $\gamma 2$ receptors, giving rise to a more prominent δ receptor contribution to tonic inhibition than our previous results at a fixed membrane potential have suggested. In contrast to the possibility of disproportionate δ receptor rectification, we found no difference in rectification properties among receptor subclasses in DGCs, so stronger rectification is unlikely to contribute to a more prominent role of δ receptors in inhibition.

In summary, our results suggest that $\delta T6Y$ is a helpful tool for exploring the role of GABA_AR subpopulations in neuronal physiology and pharmacology. Caveats related to altered agonist sensitivity have not been detectable in KI mice. Differences detected in recombinant receptors need to be considered when interpreting data from knock-in mice. However, with regard to previous results with DGCs, we see no reason for conclusions drawn to date to be revised. If the changes to baseline function are detected in other native cell types, the present results may help to circumvent the limitations.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Washington University IACUC. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

SM, JB, JS, and CZ designed research. H-JS and XL performed research. H-JS, XL, JS, CZ, and SM wrote the article.

All authors contributed to the article and approved the submitted version.

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Conflict of Interest: CZ serves on the Scientific Advisory Board to Sage Therapeutics.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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