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GABA$_A$ receptor $\alpha$5 subunits contribute to GABA$_A$,slow synaptic inhibition in mouse hippocampus

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GABA$_A$ receptor $\alpha$5 subunits and slow synaptic inhibition

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Abstract

GABA<sub>A</sub> receptor α5 subunits, which are heavily expressed in the hippocampus, are potential drug targets for improving cognitive function. They are found at synaptic and extrasynaptic sites and have been shown to mediate tonic inhibition in pyramidal neurons. We tested the hypothesis that α5 subunits also contribute to synaptic inhibition by measuring the effect of diazepam (DZ) on spontaneous and stimulus-evoked inhibitory postsynaptic currents (IPSCs) in genetically modified mice carrying a point mutation in the α5 subunit (α5-H105R) that renders those receptors insensitive to benzodiazepines. In wild type mice, DZ (1 μM) increased the amplitude of spontaneous IPSCs (sIPSCs) and stimulus-evoked GABA<sub>A,slow</sub> IPSCs (eIPSCs) and prolonged the decay of GABA<sub>A,fast</sub> sIPSCs. In α5-mutant mice, DZ increased the amplitude of a small-amplitude subset of sIPSCs (<50 pA) and eIPSCs (<300 pA) and prolonged the decay of GABA<sub>A,fast</sub> sIPSCs, but failed to increase the amplitude of larger sIPSCs and eIPSCs. These results indicate that α5 subunits contribute to a large-amplitude subset of GABA<sub>A,slow</sub> synapses, and implicate these synapses in modulation of cognitive function by drugs that target α5 subunits.

Keywords

hippocampus, synaptic transmission, GABA<sub>A</sub> receptor, diazepam, α5-H105R mouse model
Introduction

GABA<sub>A</sub> receptors (GABA<sub>A</sub>Rs) are anion-selective ion channels that underlie inhibitory neurotransmission in the brain. These receptors are assembled as pentamers from several closely related classes of subunits (α1-6, β1-3, γ1-3, δ, π, θ and ε) (McKernan and Whiting 1996). Individual subunits differ in their regional and subcellular patterns of distribution, with different subunits conferring distinct biophysical and pharmacological properties to receptors that incorporate them (Fritschy and Mohler 1995; Pirker et al. 2000). Pharmacological modulation of specific GABA<sub>A</sub>Rs assemblies has been linked to a number of behavioral effects, including sedation, anxiolysis, amnesia, and reduced seizure susceptibility (Rudolph et al. 1999; Low et al. 2000; McKernan et al. 2000).

Receptors that incorporate α5 subunits show a unique distribution in the brain. They are expressed primarily in the hippocampus, where they comprise approximately 25% of all GABA<sub>A</sub> receptors (Sur et al. 1998; Pirker et al. 2000). Studies utilizing pharmacological agents and genetic manipulations have demonstrated that α5 subunits play a role in hippocampus-dependent learning (Collinson et al. 2002; Crestani et al. 2002; Chambers et al. 2004; Dawson et al. 2006; Yee et al. 2004; Gerdjikov et al. 2008), in generating gamma oscillations (Towers et al. 2004), and in controlling hippocampal network excitability (Houser and Esclapez 2003; Scimemi et al. 2005; Glykys and Mody 2006).

Because they do play important roles in hippocampal function, the physiological nature of inhibition mediated by α5-GABA<sub>A</sub>Rs is of considerable interest. Until recently it was thought that the majority of receptors containing α5 subunits are located at extrasynaptic sites (Brunig et al. 2002). This suggested that α5-GABA<sub>A</sub>Rs underlie a non-synaptic form of “tonic inhibition” found in pyramidal cells under conditions of elevated extracellular GABA
concentration (Caraiscos et al. 2004; Scimemi et al. 2005; Prenosil et al. 2006). More recent electron microscopic studies have revealed that although many α5-subunits are located at extrasynaptic sites, they are also found at synapses on the dendrites of hippocampal pyramidal cells (Serwanski et al. 2006). Furthermore, it was recently shown that IPSPs produced by dendrite-targeting interneurons in neocortex utilize receptors that incorporate α5 subunits (Ali and Thomson 2008). These findings raise the possibility that receptors incorporating α5 subunits may also contribute to some forms of synaptic inhibition in hippocampal CA1 pyramidal neurons. In particular, α5 subunits may contribute to a form of synaptic inhibition located in the dendrites of CA1 neurons that has been termed “GABA\textsubscript{A,slow}” to reflect its distinctive activation and deactivation kinetics (Pearce 1993). However, studies addressing this issue have yielded conflicting results. Some have supported a contribution of α5 subunits to fast spontaneous and evoked IPSCs (Collinson et al. 2002), others have reported no contribution to fast spontaneous IPSCs (Caraiscos et al. 2004; Glykys and Mody 2006) but to a fraction of slow spontaneous IPSCs (Caraiscos et al. 2004; Glykys and Mody 2006), and yet others have demonstrated a partial contribution of α5 subunits to spontaneous GABA\textsubscript{A,slow} IPSCs but no contribution to evoked slow IPSCs (Prenosil et al. 2006).

If α5 subunits do indeed contribute to phasic inhibition, this would have important implications for understanding the mechanisms by which these receptors and the synapses that incorporate them influence cognitive function. Therefore, we assessed the contribution of α5-GABA\textsubscript{A}Rs to fast somatic and slow dendritic synaptic inhibition by studying genetically altered mice that carry a mutation in the α5 subunit (α5-H105R) rendering α5-containing GABA\textsubscript{A} receptors insensitive to diazepam (Crestani et al. 2002). We found that α5 subunits
do contribute to a subset of GABA_{A,slow} IPSCs characterized by large-amplitude spontaneous and evoked responses.
Materials and Methods

Mouse breeding

The generation and breeding of $\alpha$5-H105R mice has been described previously (Crestani et al. 2002). In short, a mutation in the gene of the $\alpha$5-subunit of the GABA$_A$ receptor was generated in RW-4 embryonic stem cells, which were derived from strain 129X1/SvJ and resulting mice were bread with 129X1/SvJ mice originally purchased from RCC, Füllinsdorf, Switzerland. The mutation was maintained on this “Swiss 129X1/SvJ” inbred background. For electrophysiology experiments, male offspring from homozygote or heterozygote breeding pairs were studied. Mice from heterozygote pairs were genotyped using the primers 5’ – TTAAAACCAGCACCTTTCATCTTTC and 5’ – GAGGCCACCTATGCTTCCAGCTT. Because of the inclusion of loxP in an intron adjacent to the exon harboring the H105R point mutation, the amplified fragments are approximately 50 base pairs larger in the mutant mice than in wild type.

Slice preparation

All animal procedures were performed according to a protocol approved by the University of Wisconsin Institutional Animal Care and Use Committee. Male mice, aged 24-54 days (mostly between 30-37 days), were decapitated under 3% isoflurane anesthesia. The heads were immediately immersed in cold (~0 °C) “cutting solution” which contained (in mM) NaCl 127, KH$_2$PO$_4$ 1.2, KCl 1.9, NaHCO$_3$ 26, CaCl$_2$ x 2H$_2$O 0.9, MgSO$_4$ x 7H$_2$O 2.7, glucose 10, pH 7.3 when saturated with 95% O$_2$ /5% CO$_2$ (290 -300 mOsm). The brain was glued to the tray of a vibrating microtome (Leica model VT1000S, Leica Microsystems Nussloch GmbH, Nussloch, Germany) using cyanoacrylate glue. Slices 350 or 400 $\mu$m thick
were cut in a plane ∼15° off the frontal plane, and transferred to an incubation chamber filled with artificial cerebrospinal solution (ACSF) containing NaCl 127, KH$_2$PO$_4$ 1.2, KCl 1.9, NaHCO$_3$ 26, CaCl$_2$ x 2H$_2$O 2.2, MgSO$_4$ x 7H$_2$O 1.4, glucose 10, pH 7.3 when saturated with 95% O$_2$/5% CO$_2$ (290 mOsm). Slices kept at room temperature remained in the incubation chamber for at least 1 hour before being transferred to a recording chamber perfused at 2.5-3.0 ml/min with ACSF.

**Electrophysiology**

Voltage clamp recordings were performed at room temperature using standard whole cell patch-clamp techniques (Hamill et al. 1981). Signals were amplified using a MultiClamp 700A amplifier (Molecular Devices, Sunnyvale, CA), filtered at 4 kHz, digitized at 10 kHz using a Digidata 1322A analog-to-digital converter (Molecular Devices, Sunnyvale, CA) and acquired using Clampex (Version 9.0.1.25, Molecular Devices, Sunnyvale, CA).

Inhibitory currents were investigated in isolation. To block glutamatergic transmission, D-2-amino-5-phosphonovaleric acid (AP-5, 20 μM) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM) were applied in the bath solution. Bicuculline methiodide (20 μM) or picrotoxin (100 μM) were used in a subset of experiments to confirm that responses were mediated by GABA$_A$ receptors. Pipettes were filled with an intracellular solution containing (in mM) CsCl 110, KCl 30, MgCl$_2$ 2, NaHEPES 10, BAPTA 10, Na$_2$ATP 2, Na$_3$GTP 0.3, QX-314 4, pH 7.3 (290–300 mOsm, 2.5-3 MΩ). For some experiments with evoked responses, internal solution contained (in mM) CsCl 30, K-gluconate 90, KCl 3.5, NaCl 5, NaHepes 10, EGTA 5, MgATP 4, Na$_3$GTP 0.4, Na$_2$phosphocreatine 10, QX-314 5, pH 7.2-7.3 (290-300 mOsm, 3-5 MΩ).
Electrically evoked IPSCs (eIPSCs) and spontaneous IPSCs (sIPSCs) were recorded at -60 mV from pyramidal-shaped neurons (presumed pyramidal neurons) located in the stratum pyramidale of the CA1 region of the hippocampus. Slow eIPSCs were elicited by electrical stimuli (30-200 µA at 0.033 Hz) using a glass monopolar stimulating electrode filled with 1M NaCl (tip resistance approx. 3 MΩ), placed at the border of stratum radiatum and stratum lacunosum-moleculare, at a site displaced laterally by approximately 100-250 µm from the site directly opposite the recording electrode. Current pulses (0.1 ms duration) were delivered via a constant-current stimulus isolator (model A365D, WPI Inc., Sarasota, Fl). Stimulus intensity was adjusted to evoke responses with a monophasic rise and with amplitudes ~50% of the maximal response. The access resistance was typically 5-15 MΩ, and was compensated by 50-80% using amplifier circuitry. Cells that exhibited more than 20% change in access resistance were excluded from analysis.

To compare IPSCs under control and drug conditions, analyses were restricted to the times exhibiting steady-state effects. For diazepam (DZ, 1 µM), full effects were observed approximately 8 to 10 minutes following the initiation of drug application (Fig. 5E, F, G, H). Flumazenil (FLUM, 5µM), a wide-spectrum benzodiazepine site antagonist (Hunkeler et al. 1981) or weak inverse agonist (De Vry J. and Slangen 1985; King et al. 1985), reversed the effect of DZ in approximately 5-8 minutes. For analyses we used the last 3 minutes of recordings obtained in each condition. Because the α5-H105R mutation has been reported to cause FLUM to act as a partial agonist (Kelly et al. 2002), we did not utilize measurements of IPSC amplitude in the presence of FLUM to calculate effects of DZ on IPSCs. Pharmacological effects of DZ on evoked and spontaneous IPSCs were investigated in different groups of cells.
Data analysis

Spontaneous inhibitory postsynaptic currents (sIPSCs) were analyzed using computer software (Mini Analysis 6.0.3, Synaptosoft, Inc., Decatur, GA). To detect sIPSCs, the search protocol threshold was set at 3 times the root mean square (RMS) noise level, which typically was 3-5 pA. To assess effects of DZ on fast sIPSC kinetics, for each cell (10 cells in CTRL and DZ, 4 cells in DZ+FLUM), at least 40 fast sIPSCs were averaged, normalized, and characterized by their 10-90% rise and decay times. Events were chosen based on the presence of a stable baseline level and the lack of spontaneous events during the deactivation phase, and aligned at the time of half-maximal amplitude of the rising phase. The decay phases of averaged fast sIPSCs were fitted to a bi-exponential function using a Simplex fitting algorithm. Individual sIPSCs were characterized by rise time (time to peak), and decay time (time from peak to 0.3 times the peak). Spontaneous IPSCs were considered as GABA\textsubscript{A,fast} if rise time was less than 4 ms and decay time was less than 40 ms, and GABA\textsubscript{A,slow} if rise time was greater than 4 ms and decay time was greater than 40 ms (Banks et al. 1998; Banks et al. 2002).

Slow evoked IPSCs were analyzed using Clampfit (version 9.0.1.25, Molecular Devices, Sunnyvale, CA). Responses were characterized by 10-90% rise time (t\textsubscript{rise}), and by the time constant of decay (t\textsubscript{decay}), which was obtained by fitting the decay phase to a mono- or bi-exponential function using the Levenberg-Marquardt algorithm. The accuracy of the fit was judged by eye. Evoked IPSCs were usually best fit by a single exponential function, as described previously (Banks et al. 1998). For those cases in which the current was better fit by a biexponential function, t\textsubscript{decay} was taken as the weighted time constant. Only IPSCs with a monophasic rise were used for amplitude and rise time measurements, and only IPSCs that
lacked large amplitude spontaneous currents during the decay phase were used for decay measurements.

**Statistics**

Statistical analyses were performed using GraphPad PRISM (version 5.00 and 8.00, GraphPad Software, San Diego California USA, www.graphpad.com) or Origin (version 7.5, OriginLab, Northampton, MA).

For slow evoked IPSCs, amplitude distributions were fitted with a single Gaussian function or the sum of two Gaussian functions. For slow sIPSCs, amplitude distributions were fitted with one-component and two-component log-normal functions using Origin. To achieve fit convergence, the three parameters characterizing each individual component (A-amplitude, xc-center, w-width) were evaluated iteratively while the three parameters of the other component were fixed; this procedure was repeated for each component alternately until the sum of errors was minimized. Comparisons between one-component and two-component models were performed using the F-test, with values of p<0.001 required to favor a sum of two over one component. The K-S test (Kirkman 1996) was used to compare the amplitude distributions of each component in the absence vs. presence of DZ by separating the cumulative distributions according to the ratios of their areas from the two-component log-normal fits.

The critical value for statistical significance was set at p<0.05, unless indicated otherwise. Data are presented as mean ± s.d.
Chemicals

All salts and drugs were obtained from Sigma (St. Louis, MO, USA). Stocks of diazepam (10 mM) and flumazenil (30 mM) were made in DMSO and stored in a freezer at —20 °C. The final concentration of DMSO was less than 0.1%, which was found to have no effect on GABA_A receptor mediated currents (Harney et al. 2003).
Results

GABA<sub>A,fast</sub> sIPSCs

More than 99% of spontaneous IPSCs (sIPSCs) in CA1 pyramidal neurons have kinetic and pharmacologic properties classifying them as GABA<sub>A,fast</sub> IPSCs. They are generated by somatic and perisomatic inhibitory synapses made by several different classes of inhibitory neurons including basket cells, axo-axonic cells, and trilaminar cells (Buhl et al. 1994) and they arise from receptors containing α1 and α2 subunits (Nusser et al. 1996). However, a recent publication reporting a heavy expression of the α5 subunit in stratum pyramidale of the CA1 region (Prenosil et al. 2006) prompted us to test whether GABA<sub>A,fast</sub> IPSCs may be mediated in part by receptors containing α5 subunits. Thus, we examined the characteristics of GABA<sub>A,fast</sub> sIPSCs under control conditions, and the impact of a saturating concentration of DZ (1 μM) on GABA<sub>A,fast</sub> sIPSCs in α5-H105R and in WT mice.

Under control conditions, characteristics of fast sIPSCs were similar in the two genotypes (Table 1, Fig. 1). In both cases, DZ increased the relative proportion of the slow component, and increased the weighted τ<sub>decay</sub> (Table 1, Fig. 2A, B). The similarity of fast IPSC properties under control conditions and the similarity in effects of DZ in the two genotypes indicate that α5 subunits do not contribute substantially to GABA<sub>A,fast</sub> sIPSCs.

Although spontaneous IPSCs result from the activation of predominantly synaptic receptors, synchronous activation of several adjacent synapses may also activate perisynaptic and extrasynaptic receptors (Overstreet and Westbrook 2003; Wei et al. 2003). This mechanism promotes the generation of GABA<sub>B</sub> IPSPs (Scanziani 2000), and has been proposed to underlie the slow component of fast IPSCs with bi-exponential decay kinetics (Roepstorff and Lambert, 1994). In a previous study of receptors in excised membrane
patches exposed to brief pulses of exogenously applied GABA we found that a large proportion of receptors on the somata of CA1 pyramidal neurons have slow kinetics, and are likely located at extrasynaptic sites (Banks and Pearce 2000). Reasoning that the largest sIPSCs arise from cells that form several closely-spaced synapses on a single pyramidal neuron, such as basket cells (Buhl et al. 1995), and that these would be the most likely to activate perisynaptic or extrasynaptic receptors containing α5 subunits, we compared the effects of DZ on sIPSCs with the greatest amplitudes (the largest “5%” of all sIPSCs under each condition) in WT and α5-H105R mice.

Under control conditions, the characteristics of large fast sIPSCs were indistinguishable in the two genotypes, including rise time, decay times and relative amplitudes of the individual exponential components, weighted decay rates, and the frequency of spontaneous IPSCs (Table 1). DZ did not alter the mean amplitude of large fast sIPSCs in either genotype (WT 112 ± 33% of control; α5-H105R 80 ± 36% of control, p=0.5 for both). The weighted $\tau_{\text{decay}}$ was significantly prolonged by DZ, by 50 ± 14% in WT and by 56 ± 9% in α5-H105R mice (p>0.01 for both, Table1, Fig. 2C, D). These effects did not differ between genotypes (p=0.5-0.9). These results show that receptors containing α5 subunit do not contribute substantially to either the early or late component of fast phasic inhibition.

**GABA$_{A,\text{slow}}$ sIPSCs**

Although the great majority of sIPSCs in CA1 cells arise from GABA$_{A,\text{fast}}$ synapses, a small proportion of sIPSCs have kinetic and pharmacologic characteristics that closely match those of GABA$_{A,\text{slow}}$ evoked IPSCs (eIPSCs) (Banks et al. 1998; Prenosil et al. 2006; Glykys and Mody 2006). We found that slow sIPSCs were present in both WT and α5-H105R mice
(Fig. 1). Under control conditions, they did not differ in rise time, decay, or mean amplitude in the two genotypes (p>0.05, unpaired t-test, Table 2). However, the frequency of slow sIPSCs, which ranged from 0.03 s\(^{-1}\) to 0.18 s\(^{-1}\) (mean 0.09 ± 0.01 s\(^{-1}\)) in WT mice, and from 0.03 s\(^{-1}\) to 0.11 s\(^{-1}\) (mean 0.05 ± 0.01 s\(^{-1}\)) for α5-H105R mice was significantly lower in α5-H105R mice compared to WT (p<0.05, unpaired t-test, Table 2).

To test for the presence of α5 subunits at slow synapses we compared the impact of DZ on slow sIPSCs in the two genotypes. DZ increased sIPSC frequency (WT: +54 ± 13%; α5-H105R: +51 ± 5%) modestly slowed sIPSC decay (WT: +9.6 ± 23%; α5-H105R: +9.7 ± 23%), and increased IPSC mean amplitude (WT: +37 ± 54%; α5-H105R: +13.4 ± 17%). None of these effects differed in the two genotypes. However, there was a trend toward a greater effect of DZ on amplitude in WT mice (0.05<p<0.10, Table 2).

Examination of cumulative amplitude distributions revealed that in WT mice DZ caused a shift to larger values across the entire range of IPSC amplitudes, whereas in α5-H105R mice DZ increased the amplitude of the smaller but not larger IPSCs (Fig. 3A, B). This amplitude-dependent difference in DZ modulation in cells from α5-H105R mice suggests that multiple forms of slow IPSCs may exist, with only large-amplitude synapses utilizing α5 subunits. To examine this possibility in more detail, we fit the amplitude distributions of slow sIPSC in WT and α5-H105R mice in the absence and presence of DZ. Amplitude distributions were skewed toward small-amplitude events (Fig. 3 B-E); in all cases these distributions were better fit by the sum of two log-normal distributions than by a single log-normal distribution (F-test, p<0.0001). To perform statistical comparisons of the effects of DZ on the two components in the two genotypes, we separated IPSCs into small- and large-amplitude groups according the ratios of the areas of the individual components in the two-
component log-normal fits (Table 3), and compared these distributions using the K-S test. In WT mice, DZ shifted the amplitude distribution of both the smaller and larger components to larger values (small: +45%; large: +81%, p<0.01 for both, Fig. 4A, C), whereas in α5- H105R mice, DZ shifted the amplitude distribution of the smaller component (+34.5%, p<0.01, Fig. 4B) but not the larger component (+3%, p=0.32, Fig. 4D).

We were concerned that assigning a “cutoff” between large- vs. small-amplitude sIPSCs based on the ratio of the areas of the two components (as described above) would introduce a systematic bias through misclassification of events. For example, large events would be biased most since these comprise only 40% of events in WT and 29% of events in α5-H105R mice (Table 3). Therefore, to minimize this error, we also compared the effect of DZ on only the largest 20% of IPSCs in each group. Again, we found that DZ increased amplitudes of large-amplitude sIPSCs in WT mice (+ 47%, p<0.01), but failed in α5-H105R mice (+1%, p=0.361).

These results indicate that α5 subunits are present at a large-amplitude subset of synapses that give rise to GABA<sub>A,slow</sub> sIPSCs.

**GABA<sub>A,slow</sub> eIPSCs**

To test for a possible contribution of α5 subunits to evoked GABA<sub>A,slow</sub> IPSCs, we compared the effects of DZ on responses evoked by electrical stimuli delivered to the border of stratum radiatum and stratum lacunosum-moleculare in WT vs. α5-H105R mice (Fig. 5). Under control conditions, with the stimulus intensity adjusted to elicit IPSCs with a monophasic rising phase and an amplitude approximately one-half of the maximum (e.g. 100 μA in Fig. 5C and 200 μA in Fig. 5D), there were no differences between genotypes in mean
amplitude (WT: -315 ± 160 pA, α5-H105R: -307 ± 130 pA), 10-90% rise time (WT: 6.7 ± 2.1 ms, α5-H105R: 7.4 ± 2.3 ms), or τ_{decay} (WT: 56.1 ± 22 ms, α5-H105R: 56.1 ± 16 ms). These kinetic characteristics are similar to those that we, and others, have described previously under drug-free conditions (Banks et al. 1998; Glykys and Mody 2006). DZ significantly increased the mean amplitude in WT mice, by 46 ± 9% (paired t-test, p<0.01) and slowed decay by 32 ± 17% (p<0.05). In α5-mutant mice, DZ also significantly increased the mean amplitude, by 38 ± 11% (p<0.05), and slowed decay, by 28 ± 18% (p<0.001). These average effects were not different in the two genotypes (unpaired t-test, p=0.6).

The above results, which revealed neither differences between genotypes in mean response characteristics under control conditions nor differences between genotypes in DZ modulation, would thus seem to indicate that α5 subunits do not contribute to evoked GABA_A,slow IPSCs. This conclusion would match that made by Prenosil and colleagues, who reported essentially identical effects of DZ on evoked IPSCs in WT mice (46% increase in amplitude, 29% increase in τ_{decay}), and no effect of DZ in “triple-mutant” (α1-H101R/α2-H101R/α3-H126R-mice) (Prenosil et al., 2006).

We did note, however, that under control conditions, with stimulus intensity adjusted to elicit half-maximal responses, the mean eIPSC amplitudes from individual cells fell into two groups, distinguished by amplitudes smaller or larger than 300 pA (Fig. 6A, B). In some cases, even the responses of individual cells to stimuli delivered with a constant intensity fell into two discrete and separate amplitude ranges (e.g. Fig. 5A), but this pattern was seen in a minority of cases (both in WT: 40% and mutant: 14%); more often, responses in individual cells were more uniform (e.g. Fig. 5B). This bimodal distribution was also evident when all responses evoked from all cells were pooled (i.e. twelve individual responses from each of 10
WT and 14 α5-mutant mice, for a total of 120 and 168 responses), and plotted as amplitude histograms (Fig. 6B, E). Fits of these pooled data to Gaussian distributions were significantly better using two- than one-component models (p<0.001, F-test).

To evaluate the effects of DZ on the two populations of IPSCs (i.e. small and large) in the two genotypes, we used two approaches. In the first, we separated cells into two groups based on the mean response amplitudes of eIPSCs under control conditions. In WT mice, DZ increased the mean amplitude of both small and large eIPSCs (p<0.05 for both, paired t-test, Fig. 6A, Table 4). However, in α5-mutant mice, DZ increased the IPSC amplitude of only the small-amplitude group (p<0.05, paired t-test, Fig. 6D, Table 4) but not of cells with large-amplitude responses under control conditions (p>0.05, paired t-test; Fig. 6D, Table 4). In the second approach, we compared the amplitude distributions of the pooled data that had been fitted with the sum of two Gaussian functions (plotted as cumulative amplitude distributions Fig. 6C, F) in the absence vs. presence of DZ. The patterns of DZ responsiveness for these evoked responses matched those described above for the means of eIPSCs in individual cells (Fig. 6A, D) as well as sIPSCs (Fig. 3E, F): both the small- and the large-amplitude responses were increased by DZ in WT mice, whereas in α5-mutant mice, only the small-amplitude responses were increased (Table 4). Nevertheless, DZ did slow IPSC decay of both small- and large-amplitude groups, to comparable degrees in both genotypes (WT: small +32%, large +36%; α5-H105R: small +22%, large +38%; p<0.05 for all, paired t-tests, Table 4). These findings support the conclusion that receptors containing α5 subunits contribute to a subpopulation of large-amplitude GABA<sub>A</sub>,slow IPSCs.
Discussion

The primary goal of this study was to test whether receptors containing α5 subunits contribute to GABA<sub>A</sub> receptor-mediated synaptic inhibition in mouse hippocampus. Our findings indicate that α5 subunits do contribute to a subset of GABA<sub>A,slow</sub> synaptic currents with large-amplitude evoked responses. We also found that α5 subunits do not contribute to GABA<sub>A,fast</sub> synaptic currents, even under conditions that favor neurotransmitter spillover. The results hold implications for understanding the impact of synapses that utilize receptors containing α5 subunits, and their modulation by pharmacologic agents, on cognitive function.

α5 subunits contribute to spontaneous and evoked GABA<sub>A,slow</sub> inhibition

Two lines of evidence support the contribution of α5 subunits to GABA<sub>A,slow</sub> synaptic inhibition. First, the DZ-induced increase in amplitude of the large-amplitude component of both spontaneous and electrically evoked responses was lost in α5-H105R mice; and second, the relative frequency of large-amplitude slow spontaneous IPSCs and the proportion of cells with large-amplitude evoked IPSCs were lower in α5-H105R mice than in WT mice. The first effect is directly attributable to the impact of the mutation at a key position within the benzodiazepine-binding site of α5 subunits. The mutation does not prevent receptors from responding to GABA, but it does eliminate benzodiazepine modulation of the responses. Thus, the finding that DZ modulation of large-amplitude responses was absent in mutant mice indicates that α5 subunits are present at these synapses. The second, including both the reduced proportion of large-amplitude sIPSCs and the reduced proportion of large-amplitude evoked IPSCs in mutant mice, may be attributed to a reduced number of α5 subunits in these
mice or to reduced affinity of receptors containing the α5-H105R mutation, as documented previously (Crestani et al. 2002).

Our finding that slow IPSCs in the two genotypes had similar kinetics implies that factors other than intrinsic receptor properties, such as neurotransmitter time course, determine the rate of deactivation, since reduced affinity would also be expected to accelerate deactivation if receptors are activated by transmitter transients much briefer than the synaptic responses (Jones and Westbrook 1995; Mozrzymas et al. 2003).

Previous studies that addressed the role of α5 subunits in hippocampal inhibition have consistently reported that they contribute to tonic inhibition (Caraiscos et al. 2004; Glykys and Mody 2006; Prenosil et al. 2006). However, the picture with regard to phasic inhibition has been confusing. In genetically modified mice lacking α5 subunits, spontaneous IPSCs with fast kinetics were reported to be reduced in amplitude, whereas evoked IPSCs, which had slow kinetics, were unchanged in amplitude or decay (Collinson et al. 2002). By contrast, different groups studying these same mice reported no changes in fast sIPSCs or mIPSCs, but a modest reduction in the relative frequency of sIPSCs with slow kinetics (Caraiscos et al. 2004; Glykys and Mody 2006). A recent study of mice carrying mutations in α1, α2, and α3 subunits of the GABA_A receptor that rendered them benzodiazepine-insensitive indicated that α5 subunits do contribute to slow sIPSCs, but surprisingly, not to evoked responses with similarly slow kinetics (Prenosil et al. 2006). These inconsistencies cannot be explained simply by differences in the mouse models employed, because opposing conclusions have been reached even based on the same lines of mice. Other possible explanations include the use of different criteria to separate spontaneous events into kinetically distinct groups and the use of techniques for electrical stimulation that elicited mixed GABA_A,fast and GABA_A,slow
responses. Our present results demonstrating that α5 subunits underlie a large-amplitude subset of electrically evoked synaptic currents provides another possible explanation: GABA_{A,slow} IPSCs originate from a heterogeneous population of synapses, only some of which utilize α5 subunits, and the methods used either to activate or to identify these synapses failed to distinguish between them. Indeed, the small-amplitude evoked responses that we found were unaffected by the α5 mutation (Fig. 4) were similar in amplitude (~100pA) to the evoked responses that Prenosil et al. (2006) concluded arose from non-α5 containing receptors.

**α5 subunits do not contribute to GABA_{A,fast} inhibition**

Our finding that the α5-H105R mutation alters neither the characteristics of fast IPSCs under control conditions nor their response to DZ (Table 1, Fig. 2) indicates that α5 subunits do not contribute to GABA_{A,fast} IPSCs. Other investigators reached this same conclusion by studying α5/- mice (Caraiscos et al. 2004; Glykys and Mody 2006) and α1-H101R/α2-H101R/α3-H126R mice (Prenosil et al. 2006). Our results are consistent as well with the finding that reduced α5 subunit expression in epileptic rats is associated with reduced levels of tonic GABA_{A} receptor-mediated current but no change in GABA_{A,fast} synaptic inhibition (Scimemi et al. 2005).

It was suggested previously that the slower biphasic component of fast IPSCs may result from synaptically released transmitter that spills out of synapses onto perisynaptic and extrasynaptic receptors (Roepstorff and Lambert 1994; Overstreet and Westbrook 2003). Indeed, we did find that a large number of extrasynaptic receptors with slow deactivation kinetics are present on the somata of CA1 pyramidal cells (Banks and Pearce 2000), and a
recent study reported high expression levels of α5 subunits in stratum pyramidale of the CA1 region (Prenosil et al. 2006). Therefore, although the mean properties of GABA_{A,fast} IPSCs were not altered by the α5-H105R mutation, we tested whether α5 subunits contribute to the slower component of some fast IPSCs by examining the properties of the largest fast IPSCs, which would be expected to arise from cells making the largest numbers of synaptic contacts and most likely to allow GABA to reach high levels at extrasynaptic sites. However, again we found no differences between genotypes in GABA_{A,fast} sIPSC characteristics, including the relative amplitudes and time constants of fast and slow components, under control conditions, and no difference in DZ modulation of these parameters. Therefore, we conclude that the slower decay component is not due to transmitter spillover from fast synapses onto receptors that incorporate α5 subunits, even at room temperature – a condition, which by slowing GABA uptake would be expected to accentuate spillover onto extrasynaptic receptors (Asztely et al. 1997).

The one difference in the effect of DZ on fast IPSCs in the two genotypes that we did observe was an increase in fast sIPSC frequency in α5-H105R but not in WT mice. The effect was evident as a trend for “all events”, but for the largest 5% of events, where the frequency was less variable between cells, it reached statistical significance (Table 1). The result suggests that spontaneous activity in basket cells and/or other interneurons, which generate fast sIPSCs, is under the opposing influence of multiple forms of phasic and tonic inhibition, mediated by α5- vs. non-α5-containing receptors. However, without further direct investigation of these various influences, it is difficult to deduce which are instrumental in producing this effect.
Spatiotemporal profile of neurotransmitter at GABA_{A,slow} synapses

Benzodiazepines (BZDs) have been used to estimate the degree of occupancy of postsynaptic receptors at inhibitory synapses (De Koninck and Mody 1994; Nusser et al. 1997; Perrais and Ropert 1999). The finding that the predominant effect of these agents is on the rate of decay rather than amplitude of fast IPSCs has been interpreted as an indication that receptors underlying these synaptic responses are relatively close to saturation (De Koninck and Mody 1994; Hajos et al. 2000). By contrast, receptors at perisynaptic or extrasynaptic sites that might mediate phasic responses by transmitter spillover are likely to be far from saturation. Our finding that DZ substantially increased the amplitude of both spontaneous (Table 2) and evoked GABA_{A,slow} IPSCs (Table 3), together with the previous demonstration that blocking transmitter uptake prolongs GABA_{A,slow} but not GABA_{A,fast} IPSCs (Banks et al. 2000; Prenosil et al. 2006), indicates that the spatiotemporal profile of neurotransmitter at GABA_{A,slow} synapses is quite different than that at GABA_{A,fast} synapses.

What might that spatiotemporal profile be, and what factors might contribute? Rather than existing as a cluster of low-affinity receptors that respond to a transmitter transient that is substantially briefer than the relaxation rate of activated channels (Maconochie et al. 1994), dispersed perisynaptic and extrasynaptic receptors with high affinity for GABA (Burgard et al. 1996; Yeung et al. 2003) may respond to a more slowly rising and prolonged transient whose peak concentration is only a fraction of the concentration that leads to full receptor activation. This explanation has been proposed for a slow dendritic IPSC mediated by perisynaptic δ subunit-containing receptors in dentate gyrus (Wei et al. 2003). Alternatively, release of transmitter into a high-volume cleft from which it must be cleared by GABA transporters rather than diffusion might also account for a prolonged, low-concentration
transmitter transient, as proposed for slow IPSCs in neocortical pyramidal cells that arise from neurogliaform cells (Szabadics et al. 2007). In either case, the increase in IPSC amplitude caused by DZ may provide an explanation for the increase in frequency that we observed, as a larger proportion of spontaneous IPSCs would be brought above the threshold for detection. Alternatively, it is possible that there is a true increase in the frequency of firing of interneurons that generate slow sIPSCs, produced for example by a depolarizing GABA<sub>A</sub> receptor-mediated response caused by a reversed Cl<sup>-</sup> gradient (Szabadics et al. 2006; Zhang and Jackson 1993). If this were to occur via presynaptic α5 subunits, this would provide a potential explanation for the reduced frequency of slow sIPSCs in α5-H105R mice.

**Heterogeneity of GABA<sub>A</sub>,slow IPSCs**

We focused here on the contribution of α5 subunits to GABA<sub>A</sub>,slow IPSCs and found that α5 subunits do indeed contribute. However, it is clear that other α subunits do also play a role. This is evident in the significant effect of DZ on small-amplitude sIPSCs and eIPSCs (Fig. 4, 6), and on the decay of slow sIPSCs (Tables 2, 4) in α5-H105R mice. A likely candidate is the α1 subunit. In contrast to the α2 subunit, which is found primarily at the axon initial segment (Nusser et al. 1996; Fritschy et al. 1998), the α1 subunit is found all along the somatodendritic axis, at synaptic as well as extrasynaptic sites. This suggestion is consistent with the results of a recent study of mice carrying mutations in the BDZ-binding sites of α1 and α2 subunits, which showed that DZ modulation of IPSCs evoked by distal stimulation is attenuated in α1-H101R mice, and by proximal stimulation in α2-H101R mice (Prenosil et al. 2006).
Our finding that DZ substantially increased the amplitude of the group of large sIPSCs and eIPSCs in WT but not α5-H105R mice, whereas it had similar effects on smaller sIPSCs and eIPSCs (Fig. 4, 6), suggests that α1- and α5-containing receptors are targeted to sub-populations of GABA<sub>A</sub>,<sub>slow</sub> synapses. This arrangement would be analogous to the segregation of α1 and α2 subunits at kinetically similar synapses on the somata of CA1 neurons arising from distinct classes of interneurons (Nyiri et al. 2001). Since α5 subunits preferentially associate with β3 subunits (Sur et al. 1998), and α1 with β2 (Barnard et al. 1998), and γ2 subunits are required for sensitivity to benzodiazepines (Pritchett et al. 1989), the most likely combinations would then be α1β2γ2 and α5β3γ2 receptors.

The finding that DZ slowed decay rate without altering the amplitude of large eIPSCs in α5-H105R mice (Table 4) argues against a strict isolation of α1 and α5 subunits at different classes of slow synapses. Instead, it may reflect a spatial segregation (i.e. synaptic and perisynaptic) at individual synapses that contain both receptor types, with receptors at different locations exposed to different transmitter concentrations. In this scenario, α1-containing receptors that are clustered at subsynaptic sites may be exposed to higher-concentration transmitter transients, so their modulation by DZ would result in a slowing of deactivation without a change in amplitude. By contrast, perisynaptic α1- or α5-containing receptors that are exposed to a lower concentration and slower transmitter transient would be increased in amplitude, but their decay would not necessarily be slowed - if their deactivation reflects primarily the characteristics of the transmitter transient rather than intrinsic receptor kinetics. The net response would then be a combination of these two temporally overlapping events. The higher affinity of perisynaptic α5β3γ2 receptors compared to perisynaptic α1β2γ2 receptors for GABA would then provide an explanation for their unique contribution to the
larger subpopulation of $\text{GABA}_\text{A,slow}$ IPSCs: similar transmitter transients experienced by the two receptors types would be expected to lead to stronger activation of the $\alpha_5$-containing population. At some synapses, $\alpha_1$ subunits co-localize with $\alpha_5$ subunits (Hutcheon et al. 2004), and $\alpha_1$ subunits can co-assemble with $\alpha_5$ subunits to form functional receptors (Fritschy and Mohler 1995), so it is also possible that some component of slow IPSCs arises from receptors containing both $\alpha_1$ and $\alpha_5$ subunits. Such receptors would display the pharmacological properties of the $\alpha_5$ subunit (Araujo et al. 1999).

Do different types of interneurons generate the different populations of slow eIPSCs? If so, do they utilize different complements of postsynaptic receptors, and do they play distinct functional roles in the hippocampal circuit? Although definitive answers to these questions will require paired recordings between presynaptic interneurons and postsynaptic pyramidal cells, as well as more detailed information about those interneurons’ excitatory afferents, existing data do support the possible contribution of several types of interneurons to $\text{GABA}_\text{A,slow}$ IPSCs, and of $\text{GABA}_\text{A,slow}$ IPSCs to circuit function. The axonal arbors of all three types of dendrite-targeting interneurons that are most likely to be involved (O-LM, neurogliaform, and ivy cells) are present in the vicinity of our stimulating electrode at the border of SR and SL-M (Price et al. 2005; Elfant et al. 2008; Fuentealba et al. 2008), so any of these may have been activated in our studies of evoked responses. It is certainly possible that the subunit composition at subsynaptic and perisynaptic locations differs for these different types of interneurons. It is also possible that individual neurons engage a range of receptor types, depending on its firing pattern, presynaptic modulation, and other factors that might influence transmitter release.
**Functional contributions of α5 subunits, GABA\textsubscript{A,slow} synapses, and tonic inhibition**

Several lines of evidence support a role for α5 subunits in learning and memory (Collinson et al. 2002; Crestani et al. 2002; Caraiscos et al. 2004; Yee et al. 2004; Gerdjikov et al. 2008). We suggested previously that by virtue of their position and time course, which overlap anatomically and temporally with dendritic NMDA receptors, GABA\textsubscript{A,slow} synapses...
are well suited to control synaptic plasticity (Nicoll et al. 1988; Pearce 1993; Banks et al. 2000; White et al. 2000). The present evidence that $\alpha_5$ subunits contribute to GABA$_{A,\text{slow}}$ synaptic currents is consistent with this hypothesis. However, $\alpha_5$ subunits do clearly also contribute to tonic inhibition, and play a role in controlling excitability and epilepsy (Bieda and MacIver 2004; Scimemi et al. 2005; Glykys and Mody 2006). Under which conditions of activation (tonic vs. phasic) $\alpha_5$ subunit are engaged during normal and pathological processes, and how their modulation contributes to the therapeutic actions of benzodiazepines and similar agents, remain important unresolved issues.
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Figure legends

**Figure 1.** Fast and slow spontaneous IPSCs (sIPSCs) are present in both genotypes. Examples of current traces that include both fast and slow sIPSCs are shown for eight different pyramidal cells (four WT, four α5-H105R mice). Asterisks mark slow sIPSCs. Scale bar: 100 ms, 100 pA.

**Figure 2.** Effect of diazepam (DZ, 1μM) on GABA_A,fast spontaneous IPSCs (sIPSCs) in wild type (WT) and α5-H105R mice. **A, B.** Normalized, averaged sIPSCs, from WT (CTRL τ_{fast} 6.4 ms, τ_{slow} 23 ms; DZ τ_{fast} 7.9 ms, τ_{slow} 39 ms) and α5-H105R mice (CTRL τ_{fast} 5.5 ms, τ_{slow} 26 ms, DZ τ_{fast} 6.8 ms, τ_{slow} 32 ms). **C, D.** Normalized, averaged sIPSCs derived from the largest 5% of events, from WT (CTRL τ_{fast} 6.6 ms, τ_{slow} 23 ms; DZ τ_{fast} 8.1 ms, τ_{slow} 36 ms) and α5-H105R mice (CTRL τ_{fast} 6.2 ms, τ_{slow} 32 ms, DZ τ_{fast} 7.2 ms, τ_{slow} 30 ms). Averages were derived from 42-51 events. Deactivation was fitted to biexponential functions (black curves superimposed on gray traces). Scale bar: 5 ms.

**Figure 3.** Two populations of slow spontaneous IPSCs (sIPSCs). **A, B.** Cumulative amplitude distributions of slow sIPSCs in WT and α5-H105R mouse under control conditions (thick lines) and in the presence of 1 μM DZ (thin lines). DZ failed to increase the amplitude of the largest sIPSCs in α5-H105R mice (arrow). **C-F.** Amplitude distributions of slow sIPSCs in WT and α5-H105R mice under control conditions (C, D) and in the presence of diazepam (E, F), fitted to two-component log-normal distributions. Thick lines show individual components of fits, thin lines the sum of the two components.
Figure 4. Statistical comparison of DZ modulation of small and large sIPSCs in WT and α5-H105R mice. Small (A, C) and large (B, D) slow sIPSCs were separated according to the ratios of their areas from the two-component log-normal fits (Fig. 3, Table 3) and plotted separately as cumulative probability distributions. DZ significantly increased the amplitudes of small and large sIPSCs in WT mice (A, C, K-S test, p<0.01) and of small sIPSCs in α5-H105R mice (B, K-S test, p<0.01), but did not alter characteristics of large sIPSCs in α5-H105R mice (D, K-S test, p = 0.32).

Figure 5. Effect of diazepam on GABA A,slow evoked IPSCs (eIPSCs). A, B. Responses evoked under control conditions (CTRL) and in the presence of diazepam (DZ, 1μM), in cells from wild type and α5-H105R mice. Scale bars: 50 ms, 200 pA. C, D. Response amplitude as a function of stimulus intensity for cells presented in A and B. Arrows indicate the stimulus intensity used to assess effects of DZ. Note that for the WT cell, a 100 μA stimulus evoked two populations of slow IPSCs with mean amplitudes of approximately -250 and -500 pA. E, F. Time series plots of amplitudes of slow eIPSCs, normalized to the mean of the final 12 eIPSCs under control conditions, from the cells illustrated in A and B. Each circle represents a single evoked response. The large circles designate the responses under steady state conditions that were averaged and used for further analysis. Note that for WT mice, amplitudes showed a bimodal distribution. The mean amplitudes of slow eIPSCs under control conditions were -439 pA and -534 pA for WT and α5-H105R mice, respectively. G, H. Time series plots of τ decay, normalized to the mean of the final 12 eIPSCs under control conditions, from the cells illustrated in A and B. The mean values of τ decay of slow eIPSCs in CTRL were 38 ms and 42 ms for WT and a5-H105R mice, respectively.
Figure 6. Two populations of slow evoked IPSCs (eIPSCs). A, D. Before-after plots of mean peak amplitudes of eIPSCs in individual cells, in brain slices prepared from WT mice (10 cells, A) and α5-H105R mice (14 cells, D) under control conditions (CTRL) and in the presence of diazepam (DZ, 1μM). Note that mean current amplitudes tend to fall into two distinct groups. Grey rectangles mark cells with the mean amplitudes above 300 pA. Single data points represent mean amplitudes ± s.d of small- and large-amplitude groups. B, E. Probability distributions of eIPSC amplitudes of pooled data under CTRL from WT (B) and α5- H105R mice (E), fitted with two-component Gaussian functions. C, F. Cumulative probability distributions of eIPSC amplitudes of pooled data under CTRL (thick lines) and DZ (thin lines), from WT (C) and α5-H105R mice (F), fitted with two-component Gaussian functions (superimposed grey dotted lines).
Table legends

**Table 1.** Properties of fast spontaneous IPSCs (sIPSCs) recorded under control conditions (CTRL, 10 cells), in the presence of 1 μM diazepam (DZ, 10 cells), and in the presence of DZ and 5 μM flumazenil (DZ+FLUM, 4 cells), in WT and α5-H105R mice. Data were compared using unpaired t-test. Significance: *p<0.05, **p<0.01, ***p<0.0001. The p values (statistically insignificant) for within genotypes effect of DZ vs. CTRL on sIPSCs frequency varied between 0.1 – 0.7.

**Table 2.** Properties of slow spontaneous IPSCs (sIPCSs) recorded under control conditions (CTRL, 10 cells), in the presence of 1 μM diazepam (DZ, 10 cells), and in the presence of DZ and 5 μM flumazenil (DZ+FLUM, 4 cells), in WT and α5-H105R mice. Data were compared with unpaired t-test. Significance: *p<0.05, **p<0.01. The p values (statistically insignificant) for within genotypes effect of DZ vs. CTRL on sIPSCs frequency varied between 0.1 – 0.2.

**Table 3.** Parameters of the two-component log-normal fits of amplitude distributions shown in Fig. 3 under control conditions (CTRL) and in the presence of 1 μM diazepam (DZ). A - amplitude, x_c – center, w – width. Amplitude values were used to calculate the number of small vs. large events and create cumulative distributions plots of amplitudes presented in Fig. 4.

**Table 4.** Effects of diazepam on amplitudes and decay times of slow evoked IPSCs (eIPSCs) in WT (10 cells) and α5-H105R (14 cells) mice. The mean amplitudes and time constant of
decays of small and large events was measured in groups of cells for which the mean amplitude fell below and above 300 pA respectively. Statistical comparisons were performed using paired (within genotype) or unpaired (between genotypes) t-tests. Significance: *p<0.05, **p<0.01, ***p<0.001.
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<th>Freq. (s⁻¹)</th>
<th>No. events</th>
<th>10-90% RT (ms)</th>
<th>Amplitude (pA)</th>
<th>τ₁ (ms)</th>
<th>τ₂ (ms)</th>
<th>A₁ (%)</th>
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<td><strong>CTRL</strong></td>
<td>10.8 ± 5.8</td>
<td>19349</td>
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<td>47 ± 7</td>
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**large fast sIPSCs**

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<td>7.1 ± 0.2</td>
<td>38.8 ± 1.6</td>
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<td>7.0 ± 0.5</td>
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<td>2.5 ± 0.3</td>
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<td>3.2 ± 0.3</td>
<td>106.2 ± 14.5</td>
<td>0.50 ± 0.13</td>
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<td>$60 \pm 12$</td>
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