Setting the Time Course of Inhibitory Synaptic Currents by Mixing Multiple GABA_A Receptor α Subunit Isoforms

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The kinetics of IPSCs influence many neuronal processes, such as the frequencies of oscillations and the duration of shunting inhibition. The subunit composition of recombinant GABA_A receptors (GABA_ARs) strongly affects the deactivation kinetics of GABA-evoked currents. However, for GABAergic synapses, the relationship between subunit composition and IPSC decay is less clear. Here we addressed this by combining whole-cell recordings of miniature IPSCs (mIPSCs) and quantitative immunolocalization of synaptic GABA_AR subunits. In cerebellar stellate, thalamic relay, and main olfactory bulb (MOB) deep short-axon cells of Wistar rats, the only synaptic α subunit was $\alpha 1$, and zolpidem-sensitive mIPSCs had weighted decay time constants (τ_w) of 4–6 ms. Nucleus reticularis thalami neurons expressed only $\alpha 3$ as the synaptic α subunit and exhibited slow ($\tau_w = 28$ ms), zolpidem-insensitive mIPSCs. By contrast, MOB external tufted cells contained two α subunit types ($\alpha 1$ and $\alpha 3$) at their synapses. Quantitative analysis of multiple immunolabeled images revealed small within-cell, but large between-cell, variability in synaptic $\alpha 1/\alpha 3$ ratios. This corresponded to large cell-to-cell variability in the decay ($\tau_w = 3–30$ ms) and zolpidem sensitivity of mIPSCs. Currents evoked by rapid application of GABA to patches excised from HEK cells expressing different mixtures of $\alpha 1$ and $\alpha 3$ subunits displayed highly variable deactivation times that correlated with the $\alpha 1/\alpha 3$ cDNA ratio. Our results demonstrate that diversity in the decay of IPSCs can be generated by varying the expression of different GABA_AR subunits that alone confer different decay kinetics, allowing the time course of inhibition to be tuned to individual cellular requirements.

Introduction

In most brain areas, inhibitory synapses are formed by local circuit GABAergic interneurons, a diverse population of cells that perform a multitude of functions, including the control of input summation and output generation (Klausberger and Somogyi, 2008). Interneurons generate IPSCs in their target cells by activating postsynaptic GABA_A receptors (GABA_ARs) (Farrant and Kaila, 2007), which are also the site of action of numerous clinically relevant drugs. Most of these drugs act as positive allosteric modulators of the receptors, altering phasic inhibition mainly by prolonging the decay of IPSCs (Macdonald and Olsen, 1994; Rudolph and Mohler, 2006; Sieghart, 2006).

Among central neurons the decay times of IPSCs show large variability; weighted decay times (τ_w) range from <2 ms (Bartos

et al., 2001) to >100 ms (Zhang et al., 1997). However, the exact origins of this tremendous variability are unknown. Potentially, alterations in the spatiotemporal profile of the GABA concentration in the synaptic cleft or in the properties of postsynaptic GABA_ARs could underlie this variability. However, experiments in which GABA diffusion and clearance were altered showed either no (Perrais and Ropert, 2000; Overstreet and Westbrook, 2003) or relatively little (<30%) (Williams et al., 1998) effect on miniature IPSC (mIPSC) decay. Changing the intracellular concentration of chloride from 5 to 30 mM slowed IPSC decay by ~60% (Houston et al., 2009). Changing the phosphorylation state of GABA_ARs or their association with anchoring protein complexes by blocking calcineurin (Jones and Westbrook, 1997) or protein phosphatase 1/2A (Nusser et al., 1999) activity had variable, but less than twofold, effects on the decay of IPSCs. Declustering of GABA_ARs accelerated the onset and reduced the amplitude and frequency but did not affect the decay kinetics of mIPSCs (Petrini et al., 2003; Marchionni et al., 2009). These experiments indicate that changes in the spatiotemporal profile of GABA in the synaptic cleft, the phosphorylation state of postsynaptic GABA_ARs, or the intracellular concentration of permeant ions have effects that are much smaller than the 50-fold variability in mIPSC decay times recorded from different central neurons.

Both the recording temperature and the developmental stage of the animal have a strong influence on the decay of synaptic currents. However, when recorded under identical conditions, a more than fourfold variability in τ_w remained between different cell types (e.g., $\tau_w = 10–46$ ms at 24°C) (Hájós et al., 2000) ($\tau_w =$

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3.2–14 ms at 34°C) (Nusser et al., 2001). An obvious source of this variation is the different subunit composition of postsynaptic GABA_ARs. It has been convincingly demonstrated that the subunit composition of recombinant GABA_ARs strongly affects the deactivation kinetics of GABA-evoked currents (Verdoorn et al., 1990; Macdonald and Olsen, 1994; Gingrich et al., 1995; Tia et al., 1996; Lavoie et al., 1997; Vicini, 1999; Bianchi et al., 2002; Picton and Fisher, 2007; Bright et al., 2011). However, because most central neurons express multiple subunit types (Fritschy and Mohler, 1995; Pirker et al., 2000; Wisden et al., 2002), many of which are present together in individual synapses, it is less clear how IPSC decay depends on the subunit composition of the GABA_ARs present. Here we investigate the effect of subunit composition on the τ_w by recording mIPSCs from different neuronal cell types in which the synaptic subunit content has been determined using high-resolution immunolocalization with subunit-specific antibodies.

Materials and Methods

Acute slice preparation. Male Wistar rats (age range, 21–32 d) were deeply anesthetized with either ketamine (8 mg/kg, i.p.) or isoflurane in accordance with the ethical guidelines of the Institute of Experimental Medicine Protection of Research Subjects Committee. Animals younger than postnatal day 21 were not used because GABA_AR subunit isoform expression and synaptic current kinetics are known to be altered during postnatal development. After decapitation, the brain was removed and placed in ice-cold artificial CSF (ACSF) containing the following (in mM): 230 sucrose, 2.5 KCl, 25 glucose, 1.25 NaH₂PO₄, 24 NaHCO₃, 4 MgCl₂, and 0.5 CaCl₂. Horizontal slices from the main olfactory bulb (MOB), parasagittal slices from the cerebellum, and coronal slices from the thalamus were cut at 300 μ m thickness with a vibratome (VT1000S, Leica Microsystems) and were stored in ACSF containing the following (in mM): 126 NaCl, 2.5 KCl, 25 glucose, 1.25 NaH₂PO₄, 24 NaHCO₃, 2 MgCl₂, and 2 CaCl₂. All extracellular solutions were bubbled continuously with 95% O₂ and 5% CO₂, resulting in a pH of 7.4. After a 30 min recovery period at 33°C, slices were further incubated at room temperature until they were transferred to the recording chamber.

Slice electrophysiological recordings and data analysis. Somatic whole-cell recordings were performed at 26.6 $\pm$ 0.7°C using an oblique illumination technique on an Olympus BX51WI microscope with a 40 $\times$ water-immersion objective. As synaptic current kinetics are steeply dependent on temperature, special care was taken to continuously monitor and maintain a consistent temperature during each recording session. Recordings were performed using a mixed K-gluconate- and KCl-based intracellular solution [containing the following (in mM): 65 K-gluconate, 70 KCl, 2.5 NaCl, 1.5 MgCl₂, 0.025 EGTA, 10 HEPES, 2 Mg-ATP, 0.4 Mg-GTP, 10 creatinine phosphate, and 8 biocytin; pH 7.33; 270–290 mOsm]. Voltage-clamp recordings of mIPSCs at a holding potential of –70 mV were performed in the presence of 1 μ M TTX (Alomone Labs) to block voltage-gated sodium channels and 3 mM kynurenic acid to inhibit ionotropic glutamate receptors. After establishing the whole-cell configuration and allowing for a 2 min stabilization period, an initial “baseline” period of 4 min was recorded for each cell. In a subset of cells, this was followed by the addition of 100 nM zolpidem to the bath solution. Following a 12 min period of drug equilibration, a second 4 min (“steady-state zolpidem”) period was recorded. An additional control group was recorded in the same way, except that instead of washing in zolpidem, the recording was performed in ACSF for a total of 22 min, in which the same two 4 min periods were analyzed (referred to as “baseline” and “steady-state control”).

Recordings were performed with a MultiClamp 700A amplifier (Molecular Devices). Patch pipettes were pulled with a Universal Puller (Zeitz-Instrumente Vertriebs) from thick-walled borosilicate glass capillaries with an inner filament (1.5 mm outer diameter, 0.86 mm inner diameter; Sutter Instruments). Data were digitized on-line at 20 kHz and filtered at 3 kHz with a low-pass Bessel filter. Individual mIPSCs were detected and analyzed off-line with EVAN 1.5 (Nusser et al., 2001).

Traces contaminated by the overlap of multiple synaptic currents in their rising or decaying phase were discarded. For most cell types, only mIPSCs with a 10–90% rise time of <500 μ s were included in the analysis to minimize the effects of dendritic filtering on mIPSC kinetics. For external tufted cells and nucleus reticularis thalami neurons, a 10–90% rise time cutoff value of 1.5 ms was used, as recombinant α 3 subunit-containing GABA_ARs exhibit fourfold slower activation kinetics than their α 1 subunit-containing counterparts (Gingrich et al., 1995; Picton and Fisher, 2007). This 0.5 ms rise time subselection had very little effect on the mIPSC decay. In stellate/basket cells, the τ_w of mIPSCs with 10–90% rise time <0.5 ms (3.91 $\pm$ 0.58 ms) was virtually identical to that of mIPSCs with 10–90% rise time <1.5 ms (3.97 $\pm$ 0.55 ms). Similar to the decay kinetics in control, the τ_w in 100 nM zolpidem showed practically no difference regardless of whether we used 0.5 ms (6.75 $\pm$ 1.98 ms) or 1.5 ms (6.76 $\pm$ 1.88) rise time restrictions. For deep short-axon cells (dSACs), the τ_w of mIPSCs with a 10–90% rise time <1.5 ms (5.12 $\pm$ 1.06 ms) was only 3% slower than that of mIPSCs with a 10–90% rise time <0.5 ms (4.95 $\pm$ 1.03 ms). For each cell, at least six mIPSCs were required for averaging and for the measurement of τ_w ($\tau_w = \tau_1 A_1 + \tau_2 (1 - A_1)$), where τ_1 and τ_2 are the time constants of the first and second exponential functions, respectively, and A_1 is the proportion of the first exponential function contributing to the amplitude of the IPSC. In a subpopulation of cells, peak-scaled nonstationary fluctuation analysis was used to calculate the single-channel conductance (γ) and number of channels open at the peak of the current (Nusser et al., 2001). The following criteria were used to ensure the time independence of our data during the recordings: access resistance (R_a) was subject to 70% compensation and was continuously monitored; if R_a changed >20% during the recording the cell was discarded from the analysis; all recordings were rejected if the uncompensated R_a became >20 M Ω . Furthermore, there were no correlations between uncompensated R_a , input resistance, or whole-cell capacitance and τ_w within each neuronal population. The effect of 100 nM zolpidem (see Fig. 13B) was calculated for each zolpidem-treated cell by computing the $\tau_{w(\text{steady-state zolpidem})}/\tau_{w(\text{baseline zolpidem})}$ ratio and then subtracting the average of the $\tau_{w(\text{steady-state control})}/\tau_{w(\text{baseline control})}$ ratios for all control (ACSF “treated”) cells of that neuronal type.

Visualization of the recorded cells. After recordings, slices were placed in a fixative containing 4% paraformaldehyde and ~0.2% picric acid in 0.1 M phosphate buffer (PB), pH 7.4, at 4°C for at least 24 h and then were extensively washed with 0.1 M PB and Tris-buffered saline (TBS) before further processing. For neurons recorded from the nucleus reticularis thalami, each slice was blocked in TBS with 10% normal goat serum (NGS) for 1 h and then incubated overnight in rabbit anti-parvalbumin primary antibody (Swant) diluted 1:1000 in TBS containing 0.1% Triton X-100 (TBST) and 2% NGS. Each thalamic slice was subsequently washed in TBS and then incubated in TBST containing Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (Invitrogen) diluted 1:500 and streptavidin-conjugated cyanine 3 (Cy3) diluted 1:500 (Jackson ImmunoResearch) to visualize the recorded cell. Other cell types were initially visualized with streptavidin-conjugated Cy3 (1:500 in TBST). All cells were viewed using an Olympus BX62 microscope equipped with appropriate fluorescence filters and a DP30BW charge-coupled device camera. A subset of external tufted cells was subsequently processed for three-dimensional reconstruction: slices were embedded in gelatin and resectioned at 60 μ m thickness. Biocytin was visualized using avidin–biotin–horseradish peroxidase complex followed by a reaction with 3′3′-diaminobenzidine tetrahydrochloride as chromogen and 0.01% H₂O₂ as oxidant for 12 min. Sections were then postfixed in 1% OsO₄ for 20 min, stained in 1% uranyl acetate for 25 min, dehydrated in a graded series of ethanol, and embedded in epoxy resin (Durcupan). Neurons with no obvious truncations in their dendrites were fully reconstructed with the NeuroLucida system (Micro-BrightField Europe) attached to a Zeiss Axioscope 2 microscope using a 100 $\times$ oil-immersion objective.

Heterologous expression of recombinant GABA_A receptors. Rat α 3(M) (edited form) (Ohlson et al., 2007) and γ 2L cDNAs were gifts from Marie Öhman (Stockholm University, Stockholm, Sweden). Rat α 1 was a gift from Trevor Smart (University College London, London, UK). Rat β 3 was a gift from Hartmut Lüddens (University of Mainz, Mainz, Germany). The coding region of each subunit was sequenced before use.

Figure 1. Immunofluorescent labeling for GABA_AR subunits in the main olfactory bulb. Low-magnification images of representative immunofluorescent reactions encompassing the various layers of the main olfactory bulb are shown. Bottom right, Triple labeling for the $\alpha 1$, $\beta 3$, and $\gamma 2$ subunits. Scale bar, 50 μm (applies to all panels). GL, Glomerular layer; MCL, mitral cell layer.

Figure 2. Multiple immunofluorescent labeling for GABA_A receptor subunits in the inframitral layers of the main olfactory bulb. **A**, Immunoreactivity for the $\alpha 1$ subunit (green) delineates the somatodendritic membranes of a dSAC. $\alpha 1$ -Immunopositive clusters (arrows) that also show labeling for the synaptic marker gephyrin (gray) are all immunonegative for the $\alpha 3$ subunit (red). $\alpha 3$ -Immunopositive clusters (arrowheads) are negative for the $\alpha 1$ subunit. **B**, An $\alpha 1$ -immunopositive dSAC is immunonegative for the $\alpha 2$ subunit (red). The $\alpha 2$ subunit-positive clusters originate from GCs. Immunolabeling for gephyrin (gray) indicates GABAergic synapses. $\alpha 1$ (arrows) and $\alpha 2$ (arrowheads) subunit-immunopositive puncta also contain gephyrin, demonstrating the synaptic enrichment of these GABA_AR subunits. **C**, **D**, $\alpha 1$ -Immunopositive clusters (green) in a dSAC (arrows) contain strong immunolabeling for the $\beta 2$ (**C**, red), but not for the $\beta 3$ (**D**, red), subunit. Strong $\beta 3$ -immunopositive clusters belong to GCs. Scale bars: (in **A**, **B**) **A–D**, 5 μm . **C** and **D** are in the same magnification as **A**.

HEK 293-H cells (Invitrogen) were grown in DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin (Sigma), 0.1 mg/ml streptomycin (Sigma) at 37°C, and 5% CO₂, and were maintained according to standard protocols. Recombinant $\alpha 1\beta 3\gamma 2L$ and $\alpha 3\beta 3\gamma 2L$ GABA_A receptors were expressed by cotransfecting α , $\beta 3$, and $\gamma 2L$ cDNAs at a ratio of 1:1:10, together with enhanced green fluorescent protein (0.5) using Lipofectamine 2000 (Invitrogen). An excess of $\gamma 2L$ cDNA was used to minimize the likelihood of mixed $\alpha\beta$ and $\alpha\beta\gamma$ receptors (Boileau et al., 2003, 2005). In each case, the total DNA used for transfection was 3.2 μ g. After 18–24 h, the cells were split and plated on glass coverslips. Recordings were made 24–72 h later. Dual transfection of $\alpha 1$ and $\alpha 3$ was performed at three different cDNA ratios ($\alpha 1\alpha 3$; 1:1, 1:1.5, and 1:2) while maintaining the same total DNA and the $\alpha\beta\gamma$ ratio.

Outside-out patch recording and ultrafast GABA application. HEK cells were viewed using a fixed-stage upright microscope, BX51WI (Olympus) and were perfused with “external” recording solution, containing the following (in mM): 140 NaCl, 2.5 CaCl₂, 1.2 MgCl₂, 4.7 KCl, 5 HEPES, 11 glucose; the pH was adjusted to 7.4 with NaOH. The temperature of the solution in the recording chamber ($25.8 \pm 0.6^\circ\text{C}$; $n = 54$ patches) was monitored using a miniature probe positioned close to the recording electrode (IT-21 thermocouple probe and TH-8 thermometer; Physitemp Instruments). Pipettes for outside-out patch recording were pulled from thick-walled borosilicate glass (1.5 mm outer diameter, 0.86 mm inner diameter; Harvard Apparatus) coated with Sylgard resin (Dow-Corning 184) and fire polished to a final resistance of 5–10 M Ω . The “internal” (pipette) solution contained the following (in mM): 140 CsCl, 4 NaCl, 0.5 CaCl₂, 10 HEPES, 5 EGTA, 2 Mg-ATP; the pH was adjusted to 7.3 with CsOH.

Ultrafast (1 ms duration) applications of GABA onto outside-out patches were achieved using a double-barreled application tool, made from theta glass (Hilgenberg) pulled to a tip opening of ~ 200 μ m and mounted on a piezoelectric translator (P-265.00; Physik Instrumente) attached to a micromanipulator (PatchStar; Scientifica). “Control” and agonist (1 mM GABA) solutions flowed continuously through the two barrels, and solution exchange occurred when movement of the translator was triggered by a voltage step (pClamp10; Molecular Devices). To enable visualization of the solution interface and allow measurement of solution exchange, 2.5 mg/ml sucrose was added to the agonist solution, and the control solution was diluted by 5%. The speed and duration of the solution exchange were estimated from the liquid junction current recorded from the open tip of the pipette at the end of each recording (minimum of 200 sweeps, after destruction of each patch). Liquid junction currents had a mean 10–90% rise time of 0.29 ± 0.02 ms and a mean duration (at half-height) of 0.99 ± 0.02 ms ($n = 54$). Recordings were made at -70 mV using an Axopatch 200A amplifier (Molecular Devices) (low-pass filter set at 10 kHz) and

Figure 3. mIPSC kinetics and pharmacology of main olfactory bulb dSACs. **A**, Continuous current recordings of mIPSCs from an EPL-dSAC (MDE471) before (black traces) and after (red traces) bath application of 100 nM zolpidem. **B**, Superimposed consecutive mIPSCs before (black, average in gray) and after (red, average in dark red) the application of 100 nM zolpidem. Right inset shows peak-scaled averaged mIPSCs, demonstrating that zolpidem slowed the decay of mIPSCs. Left inset illustrates a plot of mean current against current variance (peak-scaled nonstationary fluctuation analysis). Parabolic fit indicates a single-channel conductance (γ) of 31.5 pS and that 40 channels open at the peak of the averaged mIPSC ($N_p = 40$). **C**, Peak-scaled averages of baseline recordings from 14 morphologically identified EPL-dSACs (gray) and the population mean trace (cyan). τ_w of the population mean and CV are indicated. **D**, Summary of the effects of ACSF ($p = 0.37$, paired t test, $n = 4$) or 100 nM zolpidem ($p = 0.004$, paired t test, $n = 4$) on the weighted decay time of mIPSCs.

acquired using a Digidata 1440 interface board (Molecular Devices) and pClamp 10 software (Molecular Devices) at a sampling frequency of 50 kHz. Currents were analyzed using Igor Pro 6.10 (WaveMetrics) with NeuroMatic 2.6 (<http://www.neuromatic.thinkrandom.com>) after off-line filtering (eight-pole low-pass Bessel at 5 kHz; Clampfit 10; WaveM-

Figure 4. Multiple immunofluorescent labeling for GABA_A receptor subunits in the VPL and the nRT. **A**, Neither $\alpha 1$ (green) nor $\alpha 2$ (red) subunit immunoreactivity is detected in the nRT in strong gephyrin immunoreactive clusters (double arrowheads). **B**, Strong, punctate $\alpha 3$ subunit immunoreactivity (red) is observed in the nRT, showing intense colocalization with gephyrin (arrowheads). **C**, Immunoreactivity for the $\beta 1$ subunit (green) is not detected in the nRT (identified by the $\alpha 3$ immunoreactivity, red) or in the adjacent VPL, despite strong labeling in the dentate gyrus in the same section (inset). **D**, GABA_A $\alpha 1$ (red) and $\beta 2$ (green) subunits colocalize in the VPL, but are both absent from the nRT. **E**, GABA_A $\beta 3$ subunit (green) immunoreactivity in the nRT is strong and punctate, but it is much weaker in the adjacent $\alpha 1$ subunit-positive (red) VPL. **F**, **G**, High-magnification images indicate the lack of $\beta 1$ immunoreactivity in nRT neurons covered by strong $\alpha 3$ -immunoreactive puncta (**F**, arrowheads), whereas both $\alpha 3$ and $\beta 1$ subunit-immunoreactive puncta are observed on somatic profiles in hippocampal interneurons (**G**). Scale bars: **A**, **B**, **F**, **G**, 5 μ m; **C**, **D**, 50 μ m; **E**, 20 μ m. Scale bar in **A** applies to **B**. ml, Molecular layer of the dentate gyrus; VPL, ventral posterolateral thalamic nucleus.

etrics). The deactivation of GABA-evoked currents was described by fitting with the sum of two to four exponential components. The weighted time constant of deactivation ($\tau_{w,deact}$) was calculated as the sum of the time constants weighted by their fractional amplitudes. Similar results were obtained when current decay was assessed using a fit-independent method (charge transfer divided by the peak amplitude; data not shown).

Immunofluorescent reactions and analysis. Male Wistar rats (P25–P40) were deeply anesthetized with a mixture of ketamine and xylazine (0.5 ml, i.p.) and transcardially perfused with 0.9% saline followed by a fixative containing 1–2% paraformaldehyde in 0.1 M Na-acetate buffer, pH 6.0 (Lorincz and Nusser, 2010). This fixation condition not only allows the visualization of voltage-gated Na⁺ channels in axon initial segments, it also allows the visualization of the synaptic enrichment of neurotransmitter receptors without antigen retrieval. After removing the brain from the skull, brain regions were carefully dissected and sections were cut using a VT1000S vibratome (Leica). After extensive washing in 0.1 M PB, sections were blocked in TBS with 10% NGS for 1 h and then incubated overnight with a mixture of primary antibodies diluted in TBST containing 2% NGS. Combinations of the following primary antibodies were used: rabbit anti-GABA_A $\alpha 1$ (diluted 1:2000; gift from W. Sieghart, Medical University of Vienna, Vienna, Austria); mouse anti-GABA_A $\alpha 1$ (diluted 1:1000; NeuroMAB, University of California, Davis, CA); guinea

pig anti-GABA_A $\alpha 1$ (diluted 1:2000; gift from J.-M. Fritschy, University of Zurich, Zurich, Switzerland); guinea pig anti-GABA_A $\alpha 2$ (diluted 1:1000; gift from J.-M. Fritschy); guinea pig anti-GABA_A $\alpha 3$ (diluted 1:4000; gift from J.-M. Fritschy); rabbit anti-GABA_A $\alpha 4$ (diluted 1:500; gift from W. Sieghart); guinea pig anti-GABA_A $\alpha 5$ (diluted 1:1000; gift from J.-M. Fritschy); mouse anti-GABA_A $\beta 1$ (diluted 1:1000; NeuroMAB); rabbit anti-GABA_A $\beta 2$ (diluted 1:1000; gift from W. Sieghart); mouse anti-GABA_A $\beta 3$ (diluted 1:2000; NeuroMAB); rabbit anti-GABA_A $\gamma 2$ (diluted 1:2000; Synaptic Systems); mouse anti-gephyrin (diluted 1:2000; Synaptic Systems); and rabbit anti-neuroigin-2 (diluted 1:500; Synaptic Systems).

Sections were then washed in TBS and incubated in a mixture of appropriate secondary antibodies: goat anti-rabbit, donkey anti-guinea pig, and goat anti-mouse conjugated to Alexa Fluor 488 (Invitrogen), Cy3, or Cy5 (Jackson ImmunoResearch) (all diluted 1:500 in TBS containing 2% NGS) for 2 h. Slices were then washed in TBS, mounted, and viewed using an Olympus FV1000 confocal microscope. For quantitative measurements, triple labeling for the GABA_A $\alpha 1$ and $\alpha 3$ subunits and gephyrin were examined, and 29 external tufted cell somata and proximal dendrites, identified by their large diameters and juxtaglomerular somatic locations, were analyzed. Regions of interest were drawn around clusters of gephyrin immunoreactivity, and the average fluorescence in-

tensity for each cluster was calculated for both the $\alpha 1$ and the $\alpha 3$ subunits using FluoView confocal microscope software (Olympus).

All chemicals, drugs, and reagents were purchased from Sigma unless stated otherwise. All data are expressed as mean $\pm$ SD unless stated otherwise. An unpaired Student's *t* test for independent samples was used to compare within and between ACSF and zolpidem recording groups, as appropriate. Comparison between $\tau_{w,deact}$ values for different recombinant GABA_A receptors was made using Tukey's honestly significant difference (HSD) test, and segregation of $\tau_{w,deact}$ values for different recombinant GABA_A receptors was performed using *k*-means clustering; both analyses were performed using R 2.14.0 (The R Foundation for Statistical Computing; <http://www.R-project.org>).

Results

Decay kinetics of mIPSCs in neurons that express a single synaptic GABA_A α subunit type

Studies on recombinant GABA_ARs have demonstrated that α subunits have a major role in determining the affinity of GABA_ARs for GABA and for many pharmacological agents. However, the correlation between the kinetics of IPSCs and the subunit composition of synaptic GABA_ARs is less clear in nerve cells *in situ* because of the multiplicity of subunits expressed in most neuron types. Fluorescent immunolabeling experiments were therefore performed with antibodies against the $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\beta 1$, $\beta 2$, $\beta 3$, and $\gamma 2$ subunits in brain sections that were prepared in a way that allows their detection in synaptic junctions (see Materials and Methods). Antibodies to gephyrin and neuroligin-2 were used in multiple immunolabeling experiments to define GABAergic synapses (Sassò-Pognetto and Fritschy, 2000). In our reactions, we observed a lack of synaptic clustering of the $\alpha 4$ and $\alpha 5$ subunits, consistent with previous observations (Brünig et al., 2002; Crestani et al., 2002; Wei et al., 2003; Farrant and Nusser, 2005); therefore, immunoreactivity for these two subunits will not be discussed further. The patterns of immunolabeling in the CNS under our experimental conditions appeared very similar to those described previously using different fixation and antigen presentation conditions (Fritschy and Mohler, 1995; Pirker et al., 2000). However, at high magnifications, our labeling was more punctate, similar to those reactions in which antigen retrieval methods were used (Fritschy et al., 1998; Schneider Gasser et al., 2006). We first asked how the synaptic subunit composition and mIPSC decay correlate in nerve

Figure 5. mIPSC kinetics and pharmacology in identified nRT cells. **A**, Continuous current recordings of mIPSCs from an nRT cell (MDE816) before (black traces) and after (red traces) the bath application of 100 nM zolpidem. **B**, Superimposed, consecutive mIPSC traces before (black, average in gray) and after (red, average in dark red) 100 nM zolpidem application. Peak-scaled averages (right-hand inset) show the lack of effect of zolpidem. **C**, Peak-scaled averages of baseline recordings from 22 morphologically identified nRT cells (gray) and the population mean average trace (cyan). τ_w of the population mean and CV are indicated. **D**, Summary data showing the effects of ACSF ($p = 0.33$, paired *t* test; $n = 3$) and 100 nM zolpidem ($p = 0.60$, paired *t* test; $n = 5$) on mIPSC mean τ_w . **E**, A biocytin-filled nRT cell (red) is located within the parvalbumin-immunoreactive nRT (green). **F**, Higher-magnification view of the boxed region in **E**. Scale bars: **E**, 500 μ m; **F**, 100 μ m. fi, Fimbria; CPu, caudate-putamen; LGP, lateral globus pallidus; ic, internal capsule; VA, ventral anterior thalamic nucleus.

Figure 6. Multiple immunofluorescent labeling for GABA_AR subunits in the supramitral layers of the main olfactory bulb. **A**, GABA_AR $\alpha 1$ subunit immunoreactivity (green) in the glomerular layer delineates the somatic membrane of many juxtglomerular cells but does not colocalize with the $\alpha 2$ subunit (red). Punctate gephyrin labeling (gray) is associated with both the $\alpha 1$ (arrows) and $\alpha 2$ (arrowheads) subunits. **B**, GABA_AR $\alpha 1$ (green) and $\alpha 3$ (red) subunit immunoreactivity in the glomerular layer show highly variable colocalization, such that puncta on individual somatic and dendritic membranes in the overlay appear as various shades of green (cells 3 and 4), orange (cells 2 and 5), yellow (cell 1), and red (cell 6). Scale bars: **A**, 5 μ m; **B**, 10 μ m.

cells that express mainly a single α subunit type as α subunit in their synapses. Many neuronal populations were examined in the cerebral and cerebellar cortices, hippocampus, MOB, and thalamic nuclei until such cell types were identified.

In the MOB, the external plexiform layer (EPL) showed strong immunoreactivity for four α and all three β subunit isoforms (Fig. 1), consistent with the expression of multiple GABA_AR subtypes by mitral and tufted cells. In the internal plexiform layer (IPL) and granule cell layer (GCL), discrete, strongly $\alpha 1$ subunit-labeled interneurons were scattered among $\alpha 2$ - and $\alpha 3$ -immunopositive clusters of GCs. In our previous study (Eyre et al., 2008), we identified these strongly $\alpha 1$ -positive cells as deep short-axon cells with axonal projections to the EPL (EPL-dSACs). The gephyrin-immunopositive clusters on EPL-dSACs were strongly labeled for the $\alpha 1$ but were immunonegative for the $\alpha 2$ and $\alpha 3$ subunits (Fig. 2*A,B*). The $\beta 2$ subunit was found to have an expression pattern almost identical to that of the $\alpha 1$ subunit, whereas the $\beta 3$ subunit strongly colocalized with $\alpha 2$ and $\alpha 3$ subunit-immunopositive clusters and also with the nonsynaptic $\alpha 5$ subunits in GCs (Fig. 2*C,D*). The $\gamma 2$ subunit was present in virtually all gephyrin-positive clusters in the MOB. In summary, in the inframitral layers of the MOB, EPL-dSACs expressed synaptic $\alpha 1$, $\beta 2$, and $\gamma 2$ subunits, whereas GCs contained mainly the $\alpha 2$, $\alpha 3$, $\beta 3$, and $\gamma 2$ subunits in their synapses at detectable levels.

Next, we investigated the kinetic properties of unquantal mIPSCs in the $\alpha 1$, $\beta 2$, and $\gamma 2$ subunit-expressing EPL-dSACs by making whole-cell voltage-clamp recordings from morphologically verified EPL-dSACs in acute MOB slices (Fig. 3). To prevent complications in the interpretation of mIPSC decay kinetics as a result of differential dendritic filtering, we restricted our analysis to mIPSCs with 10–90% rise times <500 μ s, indicating a predominantly perisomatic origin (see Materials and Methods). In EPL-dSACs, mIPSCs occurred at a frequency of 0.50 ± 0.44 Hz ($n = 14$ cells; Fig. 3*A*) and had an amplitude of 45.0 ± 17.1 pA,

and their decay could be well fitted with the sum of two exponential functions, resulting in a τ_w of 5.0 ± 1.0 ms. Peak-scaled non-stationary variance analysis gave a weighted mean single-channel conductance of 31.5 pS (Fig. 3*B*). This value is consistent with the conductance of recombinant $\alpha 1\beta 3\gamma 2$ receptors at this temperature (Angelotti and Macdonald, 1993), indicating that the variance in the decay of mIPSCs within a single dSAC could be ascribed fully to the stochastic opening-closing behavior of the underlying channels. There was also very little variability in the τ_w among individual EPL-dSACs (coefficient of variation [CV]) of $\tau_w = 0.21$; Fig. 3*C*).

To provide another test of the subunit content underlying mIPSCs in EPL-dSACs, we used the imidazopyridine zolpidem, which has been shown to displace the benzodiazepine antagonist Ro15-1788 from $\alpha 1\beta 3\gamma 2$, $\alpha 2\beta 3\gamma 2$, $\alpha 3\beta 3\gamma 2$, and $\alpha 5\beta 3\gamma 2$ GABA_ARs with K_i values of 20, 450, 400, and 15,000 nM, respectively (Pritchett and Seeburg, 1990). Based on these recombinant GABA_AR data, we chose to use zolpidem at a concentration of 100 nM, thus providing a pharmacological tool to distinguish between $\alpha 1$ and $\alpha 2/\alpha 3$ subunit-containing receptors. Figure 3 shows recordings of mIPSCs from an EPL-dSAC during the baseline condition and after wash-in of 100 nM zolpidem. In the cell illustrated, there was a clear slowing of the decay of the averaged mIPSCs (Fig. 3*B*, insets). Across EPL-dSACs, the relative increase (50%) in τ_w following zolpidem application was significantly greater ($p = 0.004$, unpaired t test; Fig. 3*D*) than that seen following ACSF application (6%), demonstrating that zolpidem at 100 nM affects the decay of mIPSCs in the $\alpha 1$ subunit-expressing EPL-dSACs. The frequency and peak amplitude of mIPSCs were unaffected.

Interneurons in the cerebellar molecular layer (stellate and basket cells) are a well characterized neuronal population known to express the $\alpha 1$, but no other α , subunit (Vilto et al., 2008). Our immunofluorescent experiments confirmed this and demonstrated extensive synaptic colocalization of the $\alpha 1$ subunit with

$\beta 2$, $\beta 3$, and $\gamma 2$ subunits in the somata and dendrites of these cells (data not shown). We performed whole-cell recordings from 14 morphologically verified stellate/basket cells, which exhibited mIPSCs with a frequency of 0.69 ± 0.37 Hz, a peak amplitude of 61.1 ± 18.9 pA, a τ_w of 3.9 ± 0.6 ms, and a very small cell-to-cell variability in τ_w (CV = 0.15). In the subpopulation of cells treated with 100 nM zolpidem, an increase in τ_w was detected (from 3.7 ± 0.8 ms to 6.8 ± 2.0 ms; $n = 4$). This slowing of the decay was significantly different from that observed during 22 min of recording in the continuous presence of ACSF (relative increase in zolpidem, 82%; relative increase in ACSF, 39%; $p = 0.01$, unpaired t test). The decay time constant of mIPSCs and the significant effect of 100 nM zolpidem in cerebellar molecular layer interneurons are remarkably similar to those seen in EPL-dSACs. Relay cells of the ventral posterolateral nucleus of the thalamus also express only $\alpha 1$ as the α subunit, together with the $\beta 2$, $\beta 3$, and $\gamma 2$ subunits (Fig. 4D,E). Miniature IPSCs recorded from these cells also had a fast decay ($\tau_w = 6.0 \pm 2.1$ ms), demonstrating that when $\alpha 1$ is the only synaptic α subunit isoform, mIPSCs have fast, zolpidem-sensitive decay kinetics.

We next turned our attention to immunofluorescent reactions in the nucleus reticularis thalami (nRT), a brain area in which the $\alpha 3$ subunit is strongly expressed (Fritschy and Mohler, 1995). Our multiple labeling experiments demonstrated that the nRT was devoid of labeling for both the $\alpha 1$ and $\alpha 2$ subunits (Fig. 4A,B). In nRT, $\beta 1$ and $\beta 2$ subunit immunoreactivity was also absent, whereas strong punctate labeling was detected for the $\beta 3$ and $\gamma 2$ subunits (Fig. 4C–E). Recently, it has been suggested that the $\beta 1$ subunit is present in a subpopulation of nRT cells (Huntsman and Huguenard, 2006); we therefore paid special attention to the localization of this subunit. When the reactions were analyzed at low magnifications, the nRT was completely immunonegative for the $\beta 1$ subunit, but strong reactivity was detected in the hippocampal dentate gyrus in the same sections, demonstrating that our immunoreaction worked (Fig. 4C, inset). The strength and specificity of our $\beta 1$ reaction is also illustrated by the confinement of labeling to the inner half of the EPL within the whole MOB (Fig. 1). At high magnifications, $\beta 1$ subunit immunoreactivity was not detected in $\alpha 3$ -immunopositive clusters in nRT cells (Fig. 4F), despite the fact that strong synaptic clustering of the $\beta 1$ subunit was readily observed in the same $\alpha 3$ - $\beta 1$ colocalization experiments in hippocampal interneurons (Fig. 4G). Together, these experiments demonstrate that synaptic GABA_ARs in nRT cells have a subunit composition of $\alpha 3\beta 3\gamma 2$.

Recordings of mIPSCs from nRT cells indicated that mIPSCs occurred at a variable frequency (from 0.02 to 0.38 Hz) and that their amplitudes were small (19.6 ± 5.5 pA; Fig. 5A,B), consistent with previous electrophysiological data (Huntsman and Huguenard, 2000; Browne et al., 2001). The 10–90% rise times of

Figure 7. Quantitative analysis of immunofluorescent labeling of synaptic GABA_ARs in ETCs. **A**, Different ETCs show a range of $\alpha 3/\alpha 1$ subunit ratios visualized by the different color hues between green (cells 6, 7, and 8), yellow (cells 4 and 5), orange (cells 2 and 3), and red (cell 1). **B**, Plot of average $\alpha 3$ subunit against average $\alpha 1$ subunit immunofluorescence intensity of all puncta for each of 29 ETCs. Note the large heterogeneity in the intensities of both subunits, and note that cells occupy the entire parameter space; no clear clusters are evident. **C**, Distribution of the $\alpha 3/\alpha 1$ intensity ratio for 29 ETCs (black bars). Distributions of individual puncta from two cells are shown in the yellow and green histograms. As an example, the small variance (CV = 0.15) of the yellow distribution indicates that the synapse-to-synapse variability in the $\alpha 3/\alpha 1$ subunit ratio within a single cell is low. The large variance (CV = 1.35) of the total distribution shown in black demonstrates the large cell-to-cell variability in the $\alpha 3/\alpha 1$ ratio. Scale bar, 5 μ m.

these mIPSCs were considerably slower than those recorded in MOB dSACs and cerebellar stellate/basket cells, consistent with the much slower on-rate of GABA for $\alpha 3$ subunit-containing GABA_ARs (Gingrich et al., 1995; Barberis et al., 2007; Picton and Fisher, 2007). We therefore selected mIPSCs with 10–90% rise times <1.5 ms for the analysis of their decays. The τ_w of mIPSCs in nRT cells was 28.1 ± 9.8 ms (Fig. 5C), consistent with previous recordings performed at similar temperature and from animals of similar age (Huntsman and Huguenard, 2000; Browne et al., 2001). Our pharmacological experiments demonstrated that the very slow decay kinetics were unaffected by 100 nM zolpidem (relative increase in zolpidem, 2%; relative increase in ACSF, 8%; $p = 0.4$, unpaired t test; Fig. 5D). Only cells positively identified as nRT neurons by their somatic location within the parvalbumin-immunoreactive nRT (Fig. 5E,F; same cell as shown in Fig. 5A,B) were included in our analyses.

External tufted cells of the main olfactory bulb express both $\alpha 1$ and $\alpha 3$ subunits in their synapses

Having identified neuronal types that express synaptic GABA_ARs containing a single α subunit isoform and having established that

Figure 8. mIPSC kinetics and pharmacology in ETCs. **A, C**, Continuous current recordings of mIPSCs from two ETCs (left, MDE599; right, MDE542) before (black traces) and after (red traces) the bath application of 100 nM zolpidem. **B, D**, Superimposed consecutive mIPSC traces before (black, average in gray) and after (red, average in dark red) 100 nM zolpidem application. Insets show peak-scaled averaged mIPSCs of baseline (gray) and zolpidem (dark red) for each cell for comparison. Note that the decay of the fast mIPSCs in **B** is prolonged by zolpidem, whereas that of the slow mIPSC in **D** is affected to a much smaller extent. **E**, Superimposed peak-scaled averages of baseline recordings from 54 ETCs (gray) and the population mean average trace (cyan). τ_w of the population mean and the CV are indicated. **F**, Summary data showing the effects of ACSF ($p = 0.16$, paired t test, $n = 12$) and 100 nM zolpidem ($p < 0.001$, paired t test, $n = 13$) on mIPSC τ_w .

the τ_w of mIPSCs originating from $\alpha 1$ subunit-containing synapses is five to seven times faster than that seen for $\alpha 3$ subunit-containing synapses, we next turned our attention to cells that express two different synaptic α subunit types. As mentioned, in central neurons the expression of multiple GABA_AR subtypes is more of a rule than an exception, and we could therefore select from a large number of cell types for detailed investigation (e.g., from MOB mitral, MOB tufted, MOB granule, cortical and hippocampal pyramidal, cerebellar granule cells). In a recent study, Panzanelli et al. (2005) elegantly demonstrated that the $\alpha 1$ and $\alpha 3$ subunits are very strongly expressed in external tufted cells (ETCs) of the MOB; therefore, we chose this cell type and analyzed multiple labeling experiments in the superficial layers of the MOB (Fig. 6). In our reactions, we also detected both the $\alpha 1$ and $\alpha 3$ subunits in ETCs, where they showed a variable degree of colocalization. Some cells strongly expressed the $\alpha 1$ subunit, some the $\alpha 3$ subunit, and some both subunits (Fig. 6B). The $\alpha 2$ subunit is also found in the superficial MOB layers, but it showed no synaptic colocalization with the $\alpha 1$ subunit (Fig. 6A). Unfortunately, our antisera against the $\alpha 2$ and $\alpha 3$ subunits were both raised in guinea pigs, precluding their direct comparison, but we never found strongly $\alpha 2$ subunit-labeled large cells with pronounced apical dendrites, consistent with a previous study showing that the $\alpha 2$ subunit is present in GABAergic periglomerular cells (PGCs) (Panzanelli et al., 2007). Immunoreactivity for the $\beta 1$ subunit was confined to the inner half of the EPL without any detectable reactivity in ETCs (Fig. 1). Immunolabeling for the $\beta 2$ subunit was virtually identical to that for the $\alpha 1$ subunit, whereas the $\beta 3$ subunit showed extensive synaptic colocalization with the $\alpha 3$ subunit in ETCs (Fig. 1). The $\gamma 2$ subunit showed punctate labeling (Fig. 1) and had a prevailing colocalization with gephyrin.

Qualitative analysis of the colocalization reactions already suggested that ETCs do not simply form three distinct subpopulations based on their $\alpha 1$ and $\alpha 3$ subunit immunoreactivity, because there is a wide range of red, orange, yellow, and green hues in the superimposed color images (Figs. 6B, right, 7A). Such variability was also evident in cell bodies and dendritic profiles located in the outer portion of the EPL and in the juxtglomerular region. To express this finding more objectively, we quantitatively analyzed triple-labeled confocal images. We measured the fluorescence intensities of the $\alpha 1$ and $\alpha 3$ subunits using gephyrin immunofluorescence to independently delineate small regions of interest (clusters) representing putative synapses. Clusters associated with the same somatic profile were individually analyzed to calculate the within-cell, cluster-to-cluster variance. The yellow and green histograms of the $\alpha 3/\alpha 1$ intensity ratios of two representative cells in Figure 7C demonstrate the small within-cell variability from cluster to cluster. However, a much larger variability was obtained when the $\alpha 3/\alpha 1$ intensity ratios were averaged for individual cells and were plotted for all ETCs (Fig. 7C). A scatter plot of the $\alpha 1$ versus the $\alpha 3$ intensities (average of all clusters within a given cell) clearly demonstrated the lack of three distinct clusters of cells (strong $\alpha 1$, no $\alpha 3$; no $\alpha 1$, strong $\alpha 3$; strong $\alpha 1$, strong $\alpha 3$); instead, the entire space in the plot in Figure 7B was apparently filled uniformly.

Variability in mIPSC decay kinetics in external tufted cells parallels the variation in the synaptic GABA_AR $\alpha 3/\alpha 1$ intensity ratio

Next, we performed whole-cell, voltage-clamp recordings of mIPSCs from a total of 54 morphologically identified ETCs (Fig. 8). Recordings from two individual cells are shown in Figure

8A–D, demonstrating two very different mIPSC decay kinetics. The τ_w in the ETC population varied over a wide range of values (CV = 0.58; Fig. 8E); the quickest decay ($\tau_w = 3.3$ ms) was as fast as those recorded in $\alpha 1$ subunit-expressing EPL-dSACs and stellate/basket cells, whereas the slowest decay ($\tau_w = 29.8$ ms) was as slow as those recorded in $\alpha 3$ subunit-expressing nRT cells. As a result, the mean τ_w (9.3 ms) lay somewhere between the extremes seen with $\alpha 1$ -only- and $\alpha 3$ -only-expressing cell types. When we analyzed the effect of 100 nM zolpidem in a subset of the ETC population, a significant increase ($p = 0.04$, unpaired t test) in the τ_w of mIPSCs was detected (relative increase, 39%) compared with that of ACSF (relative increase, 13%; Fig. 8F). However, the effect of zolpidem was not uniform but depended on the initial τ_w of the mIPSCs. ETCs with fast mIPSC decay kinetics were highly sensitive to zolpidem (Fig. 8B), whereas ETCs with slow kinetics were not (Fig. 8D). In addition to the analysis of individual cells, the strong dependence of the zolpidem effect on the τ_w is clearly seen when all cells are plotted together (see Fig. 13B). Because cells in the juxtglomerular region of the MOB are morphologically and functionally diverse, we performed *post hoc* analysis of all our recorded cells (Fig. 9), and only identified ETCs were included in our analysis (Pinching and Powell, 1971). In a previous study from our laboratory, we identified two distinct subpopulations of ETCs based on their intrinsic electrophysiological and morphological properties (Antal et al., 2006). Keeping this heterogeneity in mind, we asked whether some of the variability in the τ_w of mIPSCs of ETCs could be the result of their morphological diversity. We found that ETCs, both with and without basal dendrites that extend into the EPL, could have either fast or slow mIPSC τ_w values (Fig. 9).

Main olfactory bulb granule cells express variable $\alpha 2/\alpha 3$ subunit ratios

In examining the immunoreactivity of the $\alpha 3$ subunit in the MOB GCL, we noticed a gradient in both the strength of the reaction and the frequency and intensity of the puncta (Fig. 10A). When we examined double $\alpha 3$ - and $\beta 3$ -immunolabeled reactions, the frequency of $\alpha 3$ subunit-immunopositive clusters that were also $\beta 3$ subunit immunopositive was much higher in the deep than in the superficial part of the GCL (Fig. 10, compare B, C). Because such a gradient was not observed for the $\alpha 2$ subunit, we predicted that the $\alpha 2/\alpha 3$ ratio varies in GCs located at different depths from the mitral cell layer in the GCL. Unfortunately, our antibodies

Figure 9. The kinetics of mIPSCs do not correlate with the dendritic morphology of ETCs. **A, B**, Neurolucida reconstructions of representative ETCs with fast mIPSC kinetics that lack (**B**, MDE758, orange cell) or that have (**A**, MDE798, green cell) basal dendrites in the EPL. **C, D**, Examples of ETCs with slow mIPSC kinetics are shown without (**C**, MDE750, cyan cell) and with (**D**, MDE795, blue cell) basal dendrites. Central inset shows peak-scaled peak-aligned averaged mIPSCs of the color-coded cells with τ_w indicated. Scale bars: 25 μ m. GL, Glomerular layer.

Figure 10. Immunofluorescent localization of the $\alpha 3$ subunit in the MOB. **A**, Low-magnification view of the distribution of the $\alpha 3$ subunit in the MOB. Note the large difference in intensity between ETCs and GCs. Within the GCL, the signal strength increases from superficial to deep zones. **B, C**, High-magnification views of double-immunofluorescent labeling for the $\alpha 3$ and $\beta 3$ subunits in superficial (**B**) and deep (**C**) GCL. Note that there are many more $\alpha 3$ subunit-immunopositive clusters in the deep GCL than in the superficial part and that all these $\alpha 3$ subunit-positive clusters colocalize with the $\beta 3$ subunit. Scale bars: **A**, 50 μ m; **B, C**, 5 μ m. Scale bar in **B** applies to **C**.

against the $\alpha 2$ and $\alpha 3$ subunits were both raised in guinea pigs, precluding the direct quantitative examination of this prediction in double-labeled reactions. We addressed the functional consequences of this potential synaptic $\alpha 2/\alpha 3$ subunit variability by recording mIPSCs under conditions identical to those of GCs

Figure 11. Properties of mIPSCs in MOB GCs. **A**, Continuous current recordings from a GC (MDE525) before (black traces) and after (red traces) bath application of 100 nM zolpidem. **B**, Superimposed consecutive mIPSCs before (black, average in gray) and after (red, average in dark red) 100 nM zolpidem application. Inset shows peak-scaled averages, demonstrating the lack of zolpidem effect on mIPSC kinetics. **C**, The population mean average mIPSC trace (cyan) is superimposed on the peak-scaled averaged mIPSCs from 48 GCs (gray). τ_w of the population mean and the CV are indicated. **D**, τ_w of the mIPSCs shows a positive correlation with the distance of the GC somata from the MCL; the deeper the somatic location, the slower the mIPSC τ_w . The correlation coefficient of a linear fit (black line) to the data is indicated. MCL, Mitral cell layer.

with somatic locations at various depths in the GCL. Miniature IPSCs had a frequency of 0.08 ± 0.05 Hz ($n = 48$ cells), a peak amplitude of 64.1 ± 21.4 pA, and a τ_w of 18.7 ± 7.3 ms (Fig. 11A–C). The mIPSC decay times showed large variability (CV = 0.38; Fig. 11C) ranging from 7.2 to 37 ms. Such large variability is consistent with variable subunit composition of postsynaptic GABA_A receptors. Because our immunofluorescent reactions demonstrated a deep-to-superficial gradient in the $\alpha 3$ subunit immunoreactivity, we plotted the τ_w of mIPSCs as a function of the somatic location in the GCL. Figure 11D demonstrates that the decay of mIPSCs in deep GCs is slower than in those located in the superficial region close to the IPL. Because the deactivation time constant of GABA-evoked currents in $\alpha 2$ subunit-

containing receptors is faster than that in $\alpha 3$ -containing receptors, this result is consistent with a proportionally higher contribution of the $\alpha 3$ subunit to synaptic receptors in deep GCs.

Transfection with different $\alpha 1/\alpha 3$ subunit ratios yields currents with wide-ranging deactivation kinetics

Although receptor deactivation following GABA removal plays a key role in determining the decay of IPSCs, little is known about how this is affected by the coexpression of mixed α subunits. To investigate this, we examined the deactivation kinetics of heterologously expressed recombinant GABA_A receptors. Specifically, we recorded macroscopic currents evoked by ultrafast (1 ms duration) application of 1 mM GABA onto membrane patches excised from HEK cells transfected with cDNAs for $\beta 3$ and $\gamma 2L$, together with $\alpha 1$, $\alpha 3$, or both $\alpha 1$ and $\alpha 3$ cDNAs at different ratios (1:1, 1:1.5, and 1:2; see Materials and Methods). In all cases, current deactivation was multiexponential, requiring two (1/54), three (29/54), or four (24/54) exponential components for an adequate fit (Fig. 12A, C). For $\alpha 1$ -only receptors, all currents ($n = 6$) were fit with three exponentials, with mean time constants of 4, 35, and 184 ms and fractional amplitudes of 58, 25, and 17%. For $\alpha 3$ -only receptors, the currents were fit with either three (1.5, 37, and 480 ms; 40, 33, and 27%; $n = 3$) or four (1.8, 27, 192, and 1050 ms; 45, 32, 12, and 11%; $n = 6$) components. Accordingly, the mean $\tau_{w,deact}$ was greater for only $\alpha 3$ subunit-containing receptors (129.0 ± 54.0 ms) compared with only $\alpha 1$ subunit-containing receptors (42.2 ± 20.5 ms; $p < 0.01$, Tukey's HSD test; Fig. 12G). Although the current decays were substantially slower than those of mIPSCs—a common observation, likely reflecting the much briefer exposure to GABA *in situ* (Overstreet et al., 2002; Mozrzymas et al., 2003) or GABA_A receptor association with synaptic proteins—the threefold difference in $\tau_{w,deact}$ was

similar to the difference in τ_w seen for $\alpha 1$ and $\alpha 3$ subunit-expressing neurons and similar to the ratio seen in other studies of recombinant α isoforms (e.g., $\alpha 1/\alpha 3\beta 2\gamma 2S$; Barberis et al., 2007; and $\alpha 1/\alpha 3\beta 3\gamma 2L$; Nimmich et al., 2009). When $\alpha 1$ and $\alpha 3$ cDNAs were cotransfected, deactivation was variable, but mean values of $\tau_{w,deact}$ increased with the proportion of $\alpha 3$ cDNA transfected (Fig. 12E–G); with $\alpha 1/\alpha 3$ ratios of 1:1, 1:1.5, and 1:2, the mean $\tau_{w,deact}$ values were 49 ± 23 , 77 ± 45 , and 98 ± 57 ms ($n = 7$, 20, and 12), respectively. With dual α subunit transfection, there was no obvious trend toward intermediate values of $\tau_{w,deact}$ in individual patches; rather, as shown in Figure 12H, values were broadly spread, falling into two groups that overlapped either the only $\alpha 1$ or the only $\alpha 3$ subunit-containing channels. At a 1:1

Figure 12. Deactivation kinetics of recombinant GABA_A receptors expressed in HEK cells following transfection of $\alpha 1$, $\alpha 3$, or both $\alpha 1$ and $\alpha 3$ cDNA. **A**, Representative current evoked by 1 ms application of 1 mM GABA to a patch from an HEK cell expressing $\alpha 1\beta 3\gamma 2L$ receptors. The black line is a superimposed triple exponential fit; the gray trace above is the residual. The inset shows the initial part of the record. **B**, The global average of normalized $\alpha 1$ currents ($n = 6$); the gray fill denotes the SEM. Inset shows the same trace on an expanded amplitude scale (record truncated) and slower time base. **C**, Same as **A** but for a current evoked in a patch from an HEK cell expressing $\alpha 3\beta 3\gamma 2L$ receptors (black line is fit with four exponential components). **D**, Global average from nine $\alpha 3\beta 3\gamma 2L$ patches (inset, same as in **B**). **E**, Three representative currents evoked in patches from HEK cells transfected with $\alpha 1$ and $\alpha 3$ cDNAs at a ratio of 1:1.5. The inset shows the average currents on an expanded amplitude scale (records truncated) and slower time base. **F**, Global averages of normalized currents for $\alpha 1$ and $\alpha 3$ cotransfected at ratios of 1:1, 1:1.5, and 1:2 ($n = 7, 20$, and 12, respectively). Inset shows the average currents on a different scale (same as in **B, D**), together with the $\alpha 1$ (blue) and $\alpha 3$ (red) averages for comparison. **G**, Box plots showing $\tau_{w, deact}$ for different α subunits. Boxes indicate 25–75th percentiles, and whiskers indicate the 10–90th percentiles; horizontal red bars denote median, and crosses denote mean values. Green circles indicate individual values. Asterisks indicate significant difference from $\alpha 3\beta 3\gamma 2L$ receptors (** $p < 0.01$, Tukey's HSD test). **H**, Plot showing $\tau_{w, deact}$ values for all patches. For those cells transfected with both $\alpha 1$ and $\alpha 3$ cDNAs (open symbols), k -means clustering of pooled data identified two clusters (open blue and open red symbols) with means of 52 and 139 ms. These clusters overlapped with the $\alpha 1$ -only (filled blue symbols) and $\alpha 3$ -only groups (filled red symbols), respectively.

transfection ratio, the kinetics remained $\alpha 1$ subunit like (100%; 7/7 patches), but the distribution shifted to 70% $\alpha 1$ subunit like (6/20 patches) and 50% $\alpha 1$ subunit like (6/12 patches) at transfection ratios of 1:1.5 and 1:2, respectively. These results suggest that differential expression of the $\alpha 1$ and $\alpha 3$ subunits could generate the range of mIPSC decay times seen in cells that express only these subunits.

Discussion

In the present article, we describe a strategy allowing the decay of inhibitory synaptic currents to be set through the expression of different amounts of two synaptic GABA_A α subunit isoforms, which on their own confer distinct decay kinetics to the IPSCs. By adopting this strategy, MOB ETCs are able to generate mIPSCs with a wide repertoire of kinetics, with τ_w ranging from 3 to 30 ms. These values span the full spectrum of τ_w , encompassing values obtained from $\alpha 1$ subunit-expressing cerebellar stellate/basket cells (4 ms) and those of $\alpha 3$ subunit-expressing nRT cells (28 ms; Fig. 13).

Differences in agonist affinity, gating, and pharmacological properties have been clearly observed when systematically varying the α subunit of recombinant GABA_ARs (Verdoorn et al., 1990; Macdonald and Olsen, 1994; Gingrich et al., 1995; Tia et al., 1996; Lavoie et al., 1997; Vicini, 1999; Bianchi et al., 2002; Picton and Fisher, 2007; Bright et al., 2011). Building on this, we have investigated the role of different GABA_A α subunits in determining the time course of IPSCs in various neuronal types *in situ* that express only a single type of GABA_A α subunit. Using high-resolution immunolocalization experiments with subunit-specific antibodies, we were able to identify three cell types in which the $\alpha 1$ subunit, and one in which the $\alpha 3$ subunit, is the

only synaptic α subunit type. The decays of the mIPSCs in the three $\alpha 1$ subunit-expressing cell types (cerebellar interneurons, thalamic relay cells, and MOB EPL-dSACs) were remarkably similar to each other (4.0 ± 0.6 , 6.0 ± 2.1 , and 5.0 ± 1.0 ms) but were very different from the $\alpha 3$ (28.1 ± 9.8 ms) subunit-expressing nRT cells. It is important to stress that the actual decay values are highly temperature dependent; these values would be approximately halved at physiological temperatures, consistent with data obtained in only $\alpha 1$ subunit-expressing hippocampal interneurons (2 ms; Bartos et al., 2001) and with data obtained in nRT cells recorded at physiological temperatures (22 ms; Huntsman and Huguenard, 2000). These results are consistent with the notion that the primary determinant of the decay of GABA_A-mediated IPSCs is the receptor subunit composition. We would like to emphasize that we have no intention to suggest that the spatiotemporal profile of GABA in the synaptic cleft (Auger et al., 1998; Williams et al., 1998) and the phosphorylation state of the postsynaptic receptors (Jones and Westbrook, 1997; Nusser et al., 1999) do not affect the decay of the IPSCs. However, it is clear that the less than twofold effect of these mechanisms is much less pronounced than the sevenfold difference seen between cells that express different α subunits.

Our data regarding the decay of mIPSCs generated at $\alpha 1\beta 2\gamma 2$ and $\alpha 3\beta 3\gamma 2$ GABA_A-containing synapses are consistent with our experiments using ultrafast GABA application on outside-out patches containing recombinant GABA_ARs containing different α subunits. Because the deactivation properties of receptor populations formed when both $\alpha 1$ and $\alpha 3$ subunits are expressed were unknown, we performed experiments in which we varied the cDNA ratio of the $\alpha 1$ and $\alpha 3$ subunits. Our results with mix-

Figure 13. Summary of the kinetics and zolpidem sensitivity of mIPSCs recorded from a variety of nerve cells under identical conditions. **A**, Peak-scaled, peak-aligned population average traces demonstrating the different kinetics underlying different GABA_A α subunit expression in different cell types. Note that the ETC average (bright green) falls between the other population averages (i.e., red-orange and blue-black). Two individual ETCs with fast (MDE578, light green) and slow (MDE795, dark green) decay kinetics are also shown. Similarly, fast (MDE950, light blue) and slow (MDE890, dark blue) individual GCs are shown. The two individual cells (GC and ETC) represent the ~15th and ~85th percentiles of the population. **B**, Plot of the effect of 100 nM zolpidem as a function of the initial τ_w of mIPSCs. Zolpidem strongly affects the decay of mIPSCs in cells with fast kinetics [cerebellar interneurons (INs), MOB EPL-dSACs] but has no effect on cells with slow kinetics (nRT cells). The effect of zolpidem on the decay of MOB ETC mIPSCs depends on the initial decay time constant of the mIPSCs.

tures of recombinant receptors mimicked our data from MOB ETC synapses, demonstrating that any deactivation time constant between that of pure $\alpha 1$ or pure $\alpha 3$ subunit-containing receptors can be produced by varying the relative amounts of these two subunits. Importantly, as in MOB ETCs seen to express both $\alpha 1$ and $\alpha 3$ subunits, we cannot make conclusions regarding the presence of mixed populations of only $\alpha 1$ and only $\alpha 3$ subunit-containing receptors or the presence of receptor populations containing both $\alpha 1$ and $\alpha 3$ subunits (Duggan et al., 1991; Benke et al., 2004) or indeed of receptors with varied subunit arrangements (Minier and Sigel, 2004). Nevertheless, our results suggest that variations in the expression of the $\alpha 1$ and $\alpha 3$ subunits can account for the range of mIPSC decay times seen in cells that express only these subunits. Although A-to-I RNA editing of the $\alpha 3$ subunit is reported to speed current deactivation (Nimmich et al., 2009), it is unlikely to contribute to heterogeneous IPSC kinetics, as editing is nearly complete at the ages studied here (Ohlson et al., 2007). One difference between the subunit

composition of postsynaptic GABA_ARs in MOB EPL-dSACs versus cerebellar stellate/basket cells and thalamic relay cells is the absence versus the presence of the $\beta 3$ subunit. We observed small differences in the decay of mIPSCs of these cell types, but the presence or absence of the $\beta 3$ subunit did not correlate with the mIPSC decay times, suggesting that it is not the presence or the absence of the $\beta 3$ subunit that is responsible for the small difference in their mIPSC decay times.

Identifying the influence of $\alpha 1$ versus $\alpha 3$ subunit-containing postsynaptic GABA_ARs on the decay of IPSCs raises the issue of whether all nerve cells could fulfill their functional requirement with either very fast (4–5 ms) or very slow (28 ms) IPSCs. Thus far, no other cell type has been reported in which the cell-to-cell variability in the decay of IPSCs spans this whole spectrum of kinetics (4–28 ms). For example, the slowest τ_w among cerebellar interneurons was 5 ms, whereas it was 6.7 ms in EPL-dSACs. These values are much smaller than that of the fastest decaying IPSCs in nRT cells (16 ms). Thus, if every nerve cell only expressed either the $\alpha 1$ or the $\alpha 3$ subunit, it would be impossible to generate the whole intermediate range of decay times (from 7 to 16 ms). To circumvent this problem, we believe that nerve cells use the strategy of expressing multiple synaptic GABA_A subunit isoforms, with either a constant or a variable ratio from cell to cell.

Do all nerve cells that express multiple synaptic α isoforms show variable expression levels, as ETCs do? In the present work, we provided another example, showing that MOB GCs have variable synaptic $\alpha 2/\alpha 3$ subunit ratios, with a correspondingly large variance in the τ_w of mIPSCs. However, our previous results in hippocampal pyramidal cells (PCs) indicate that this is not always the case. The axon initial segments (AISs) of PCs receive GABAergic input from axo-axonic cells (Klausberger and Somogyi, 2008), and it has been shown that these synapses contain both the $\alpha 1$ and the $\alpha 2$ subunits (Nusser et al., 1996; Fritschy et al., 1998). When we performed colocalization of these two subunits and compared the variability in the $\alpha 1/\alpha 2$ ratio between the AISs of different PCs, we did not find any noticeable difference. Thus, our data indicate that most hippocampal PCs use a similar $\alpha 1/\alpha 2$ subunit ratio in their AIS synapses, unlike ETCs and GCs of the MOB. Like hippocampal PCs, MOB ETCs, and GCs, the majority of neuron types in the CNS express multiple GABA_A α subunit isoforms in their synapses (Fritschy and Mohler, 1995; Pirker et al., 2000; Wisden et al., 2002). Therefore, it will be interesting to see whether they use a strategy similar to hippocampal PCs or to MOB ETCs with respect to the cell-to-cell variability of their synaptic GABA_A subtype ratio.

It has been demonstrated that some nRT cells express the $\beta 1$ subunit mRNA and that a fraction of their spontaneous IPSCs have larger peak amplitudes and faster decay kinetics (Huntsman and Huguenard, 2006). In our present work, we could not detect $\beta 1$ subunit immunoreactivity in the nRT and did not find large amplitude, fast-decaying mIPSCs in any of our recorded and identified nRT cells. The lack of a subunit cannot be concluded from the lack of immunoreactivity, but the strong synaptic labeling of MOB mitral cells and hippocampal interneurons for the $\beta 1$ subunit indicates that if $\beta 1$ subunit is present in nRT cells, its density is very low. Our data are consistent with the differences in kinetics observed previously between $\alpha 3$ subunit-expressing nRT cells and adjacent $\alpha 1$ subunit-expressing thalamic relay cells (Browne et al., 2001; Mozrzymas et al., 2007).

External tufted cells of the MOB have become the focus of much attention in recent years (Belluscio et al., 2002; Hayar et al., 2004a,b; Antal et al., 2006), leading to greater understanding of

their intrinsic properties, synaptic connectivity, and functional role in odor processing. These cells receive GABAergic inputs from local interneurons, including PGCs, superficial short-axon cells, and GCs. Although it has not been demonstrated directly, it is very likely that a single ETC receives GABAergic input from multiple sources. For example, those ETCs that possess basal dendrites should receive innervation from both GCs and PGCs onto their basal and apical dendrites, respectively. The very small synapse-to-synapse variability in the $\alpha 3/\alpha 1$ subunit ratio within a given ETC indicates the lack of synaptic input-specific regulation of postsynaptic GABA_AR subunit composition within individual ETCs. However, at the population level, the wide spectrum of IPSC kinetics present in different ETCs will allow them to play different roles in the trade-off between coding information for rapid, low threshold detection and the slower, integrative discrimination of odors.

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