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Cell type- and synapse-specific variability in synaptic $GABA_A$ receptor occupancy

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Abstract

The degree of postsynaptic type A γ-aminobutyric acid receptor ($GABA_A$ receptor) occupancy was investigated by using the benzodiazepine agonist zolpidem. This drug increases the affinity of $GABA_A$ receptors for γ-aminobutyric acid (GABA) at room temperature (Perrais & Ropert, 1999, *J. Neurosci.*, **19**, 578) leading to an enhancement of synaptic current amplitudes if receptors are not fully occupied by the released transmitter. We recorded miniature inhibitory postsynaptic currents (mIPSCs) from eight different cell types in three brain regions of rats and mice. Receptors in every cell type were benzodiazepine sensitive, as 10–20 μM zolpidem prolonged the decays of mIPSCs (151–184% of control). The amplitude of the $GABA_A$ receptor-mediated events was significantly enhanced in dentate granule cells, CA1 pyramidal cells, hippocampal GABAergic interneurons, cortical layer V pyramidal cells, cortical layer V interneurons, and in cortical layer II/III interneurons. An incomplete postsynaptic $GABA_A$ receptor occupancy is thus predicted in these cells. In contrast, zolpidem induced no significant change in mIPSC amplitudes recorded from layer II/III pyramidal cells, suggesting full $GABA_A$ receptor occupancy. Moreover, different degrees of receptor occupancy could be found at distinct GABAergic synapses on a given cell. For example, of the two distinct populations of zolpidem-sensitive mIPSCs recorded in olfactory bulb granule cells, the amplitude of only one type was significantly enhanced by the drug. Thus, at synapses that generate mIPSCs, postsynaptic receptor occupancy is cell type and synapse specific, reflecting local differences in the number of receptors or in the transmitter concentration in the synaptic cleft.

Introduction

A packet of neurotransmitter released from a presynaptic terminal evokes variable responses in a postsynaptic cell. The trial-to-trial variability in the postsynaptic response at a single release site mainly originates from the stochastic behaviour of the channels if postsynaptic receptors are fully occupied by the released transmitter (Faber *et al*., 1992) or from variations in the concentration of neurotransmitter in the synaptic cleft if receptor saturation does not occur (Frerking *et al*., 1995). Thus, a maximal receptor occupancy, i.e. receptor saturation, will result in a lower coefficient of variation (CV) of the postsynaptic responses. Indeed, a low CV was shown for inhibitory postsynaptic potentials or currents (IPSPs or IPSCs) evoked with focal stimulation in principal cells of the hippocampal formation (Edwards *et al*., 1990; Ropert *et al*., 1990; Nusser *et al*., 1998). Considering the high maximal open probability of type A γ-aminobutyric acid ($GABA_A$) receptors ($P_{o,max} = 0.6–0.8$; Jones & Westbrook, 1995; Auger & Marty, 1997; Perrais & Ropert, 1999), saturation of $GABA_A$ receptors by released γ-aminobutyric acid (GABA) was inferred at these synapses. The effect of benzodiazepines on miniature inhibitory postsynaptic currents (IPSCs), events that occur spontaneously in an action potential-independent manner, also supported this conclusion. After application of benzodiazepine agonists, modulators of $GABA_A$ receptors were thought to increase receptor affinity (Macdonald & Olsen, 1994; Lavoie & Twyman, 1996; Mellor & Randall, 1997; Perrais & Ropert, 1999), the decays of mIPSCs were prolonged with no change in their amplitude at physiological temperature (Otis & Mody, 1992; Soltesz & Mody, 1994; Poisbeau *et al*., 1997). However, the degree of receptor occupancy and the use of benzodiazepines for determining postsynaptic $GABA_A$ receptor occupancy remained controversial. Using rapid agonist applications to outside-out patches and recording the effect of benzodiazepines on mIPSCs at room temperature, an incomplete $GABA_A$ receptor occupancy was suggested in various preparations (Frerking *et al*., 1995; Galarreta & Hestrin, 1997; Defazio & Hablitz, 1998). In addition, two studies suggested that postsynaptic $GABA_A$ receptor occupancy may vary within a single cell and that the occupancy of the receptors is inversely related to the initial size of the synaptic current (Auger & Marty, 1997; Nusser *et al*., 1997). A recent study (Perrais & Ropert, 1999) using fast application of GABA to outside-out patches, has demonstrated that at room temperature the benzodiazepine agonist zolpidem (1–10 μM) increased the amplitude of currents elicited by subsaturating concentrations of GABA (100–300 μM), but not by saturating GABA concentrations (10 mM). The increase in amplitude was a direct consequence of the change in the number of $GABA_A$ receptors that bind GABA without changing the single channel conductance or the maximal open probability of the receptors. Using zolpidem at room temperature, Perrais & Ropert (1999) demonstrated that $GABA_A$ receptors are not saturated by the synaptically released GABA in rat layer V pyramidal cells. They have also shown that

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zolpidem has an anomalous effect at physiological temperatures, making it inadequate as a tool to determine $GABA_A$ receptor occupancy at these temperatures. In light of these results, we have systematically studied the degree of postsynaptic $GABA_A$ receptor occupancy by examining the effect of 10–20 μM zolpidem on mIPSCs recorded from eight different cell types in three brain regions at room temperature.

Materials and methods

Slice preparation

Male mice (15–38 days old, C57/Bl 6) were deeply anaesthetized with either ether or halothane and were decapitated. Male Wistar rats (16–20 days old) were anaesthetized with sodium pentobarbital (70 mg/kg, i.p.) and then decapitated. After opening of the skull, the brain was removed and immersed into ice-cold (~ 4 °C) modified artificial cerebrospinal fluid (ACSF), which contained (in mM): NaCl, 126; KCl, 2.5; $NaHCO_3$, 26; $CaCl_2$, 0.5; $MgCl_2$, 5; NaH_2PO_4, 1.25; glucose, 10. Coronal slices of the hippocampus and primary visual cortex, and sagittal slices of the main olfactory bulb (300–350 μm in thickness) were prepared using a Lancer Series 1000 Vibratome. The slices were incubated in ACSF [containing (in mM): NaCl, 126; KCl, 2.5; $NaHCO_3$, 26; $CaCl_2$, 2; $MgCl_2$, 2; NaH_2PO_4, 1.25; glucose, 10] for 30 min at 32 °C, followed by incubation at room temperature (22–23 °C).

Whole-cell recordings and data analysis

Whole-cell voltage-clamp recordings were obtained from neurons visualized using infrared DIC (Zeiss, Axioscope) videomicroscopy. Cell types in the hippocampus and neocortex were identified based on the location of their soma and their morphology (shape of the soma and the origin of the primary dendrites). The identity of the cells was *post hoc* confirmed after the development of biocytin. Patch electrodes were pulled from borosilicate glass capillaries with an inner filament (KG-33, 1.5 mm O.D.; Garner Glass, Claremont, CA) using either a two-stage vertical Narashige PP-83 or a Sutter P-87 puller, and had resistances of 2–8 MΩ when filled with the intracellular solution. Intracellular solution was prepared from Omnisolve water (EM Science, Gibbstown, NJ, USA) and contained (in mM): CsCl, 140; NaCl, 4; HEPES, 10; $MgCl_2$, 1; Mg-ATP, 2; EGTA, 0.05 at pH 7.2–7.3 adjusted with CsOH. In some cases, 0.3–0.5% biocytin (Molecular Probes) was added to the solution. The final osmolarity was 285–300 mOsm.

During experiments, slices were superfused continuously with oxygenated (95% O_2 : 5% CO_2) ACSF containing 2–5 mM kynurenic acid (Sigma) and 0.5–1 μM tetrodotoxin (TTX, Alomone Labs) to block ionotropic glutamate receptors and voltage-gated sodium channels, respectively. All experiments were performed at room temperature (22–23 °C). Recordings were made with an Axopatch 200B amplifier (Axon Instruments), digitized at 88 kHz (Neurocorder, NeuroData, New York) and stored on videotape, or digitized at 44 kHz and stored on a DAT recorder (DTR-1202, Biologic, Claix, France). The data were filtered at 2 kHz (eight-pole Bessel, Frequency Devices 902 or FLA-01, Cygnus Technology, Fredericton, Canada), digitized at 5–20 kHz (National Instruments LabPC+A/D or PCI-MIO-16E-4 board), and were analysed using either the Strathclyde Electrophysiology Software (courtesy of Dr J. Dempster) or an in-house software written in LabView (National Instruments, Austin, TX, USA). The threshold for event detection was set to two to three times the signal-to-noise-ratio, where the noise (3–4 pA) was the standard deviation of the baseline recorded before the events. Series resistance and whole-cell capacitance were estimated by correcting the fast current transients evoked at the onset and offset of 8 ms 5 mV voltage-command steps, and were checked every 2 min during the recording. If the series resistance increased by more than 25%, the recording was discontinued.

Amplitudes, 10–90% rise times, 50% or 67% decay times, inter-event intervals were measured for each IPSC. The decays of the averaged currents were fitted with a single or the sum of two exponential functions. A weighted decay time constant (τ_w) was calculated as $\tau_w = \tau_1 \times A_1 + \tau_2 \times (1 - A_1)$, 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. The Kolmogorov–Smirnov (K–S) test was used to compare two cumulative distributions, and Student's paired *t*-test was used to compare the changes in the mean conductance, rise time, decay time and frequency after zolpidem application. Data are presented as mean ± SEM.

Anatomical identification of neurons

After the recordings, slices were fixed overnight in 4% paraformaldehyde, 0.05% glutaraldehyde and 15% picric acid in 0.1 M phosphate buffer (PB, pH 7.4). The slices were then incubated in cryoprotecting solution (0.1 M PB containing 12% glycerol and 25% sucrose) for 1 h, freeze-thawed once in liquid nitrogen, and treated with 0.5% H_2O_2 in 0.1 M PB for 30 min to reduce endogenous peroxidase activity. Recorded neurons were visualized using avidin–biotinylated horseradish peroxidase complex reaction (ABC, Vector, Burlingame, CA, USA) with nickel-intensified 3,3′-diaminobenzidine (Sigma) as chromogen (dark blue reaction product). The slices were then re-sectioned at 80 μm thickness with a Vibratome, followed by dehydration and embedding in Durcupan.

Reagents

Bicuculline methiodide (Sigma) was applied by bath perfusion in final concentrations of 10 and 30 μM. Zolpidem (Tocris) was either dissolved in ethanol (100 mM stock solution) or polyethylene glycol (20 mM stock solution), and was diluted to the final concentration of 10 or 20 μM.

Results

In the presence of kynurenic acid (2–5 mM) and TTX (0.5 or 1 μM), spontaneously occurring inward currents were observed at holding potentials ranging between –60 and –70 mV using high Cl^--containing intracellular solution in eight different cell types of three distinct brain areas of mice ($n = 48$, P15–36) and from hippocampal CA1 pyramidal cells and CA1 GABAergic interneurons of young rats ($n = 12$, P16–20). Recorded currents were completely blocked by the $GABA_A$ receptor antagonist bicuculline methiodide (10–30 μM, data not shown), indicating that the TTX-resistant synaptic currents (mIPSCs) were mediated by $GABA_A$ receptors. The frequency of mIPSCs varied between different cell types and had a range of 0.4–5.4 Hz.

$GABA_A$ receptor occupancy in the hippocampus and dentate gyrus

We first addressed possible differences between the properties of mIPSCs in rat and mouse neurons in the hippocampal CA1 area. Two representative CA1 pyramidal cells are shown in Fig. 1. There were no significant differences in the frequency, peak conductance and weighted decay time of mIPSCs recorded in CA1 pyramidal cells between the two species ($P > 0.05$, Mann–Whitney *U*-test). In

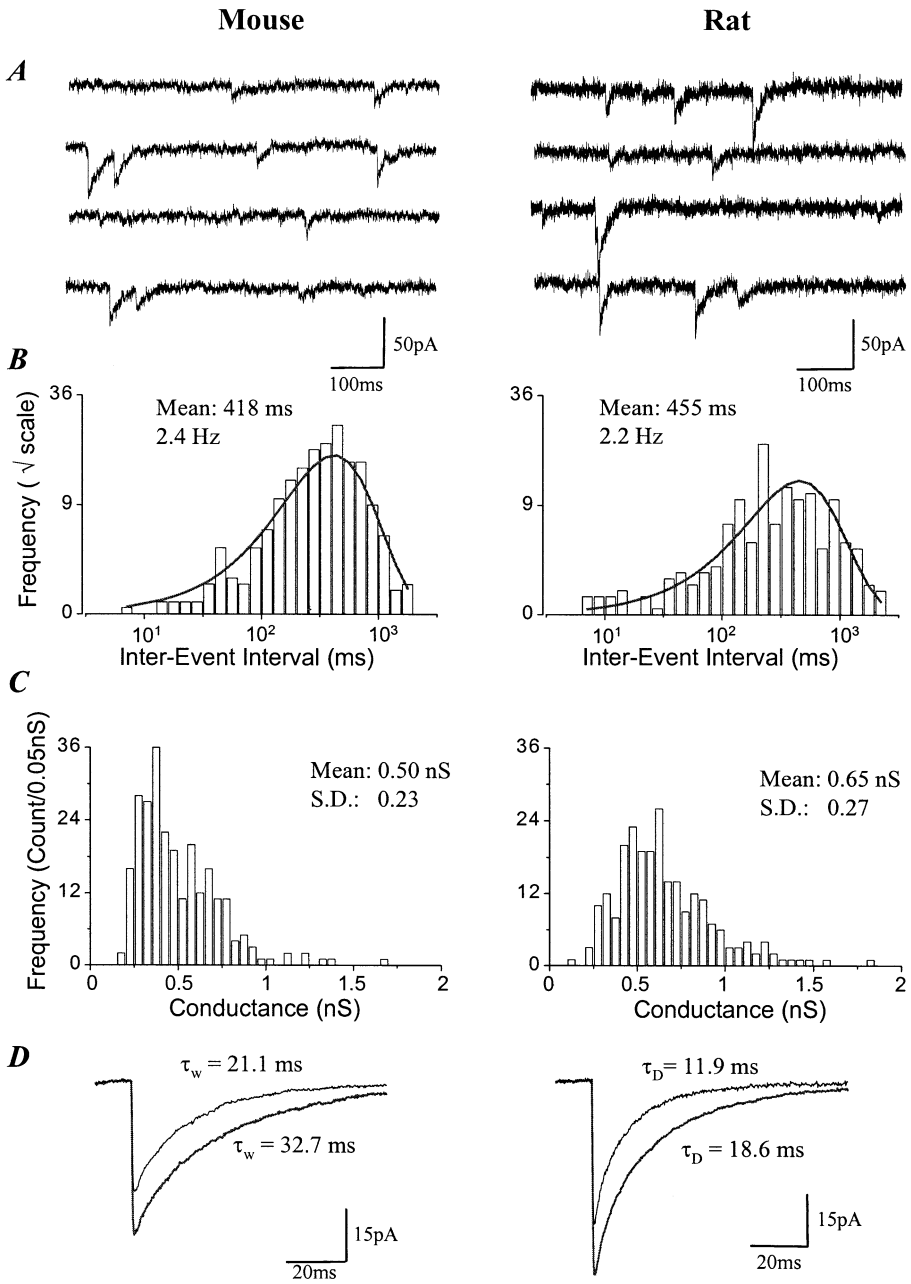

FIG. 1. Comparison of mIPSCs recorded at room temperature from CA1 mouse (P35) and rat (P18) hippocampal pyramidal cells. (A) Whole-cell patch-clamp recordings from CA1 pyramidal cells held at –65 mV in the presence of 2 mM kynurenic acid and 0.5 μM TTX. Miniature IPSCs are inward currents as the intracellular solution contained 135 mM CsCl. (B) Log-binned (10 bins per decade) inter-event intervals are plotted on a square root ordinate and illustrate similar mIPSC frequencies in pyramidal cells of mouse and rat. The fitted lines represent exponential probability density functions with means of 418 and 455 ms, corresponding to an average mIPSC frequency of 2.4 and 2.2 Hz, respectively. (C) In both neurons, the distributions of mIPSC conductances are skewed toward large values and have similar means and standard deviations ($n = 252$ in mouse and $n = 238$ in rat, respectively). (D) The decay time constant of averaged mIPSCs is faster in rats than in mice. In the mouse, the decay of the averaged mIPSC is best described by the sum of two exponentials [$\tau_1 = 10.7$ ms (36%), $\tau_2 = 26.9$ ms, $\tau_W = 21.1$ ms], whereas in the rat neuron it is adequately described by a single exponential function ($\tau_D = 11.9$ ms). Application of 10 μM zolpidem (thick traces) increased the amplitude (by 35 and 31% in mouse and rat, respectively) and the duration (by 55 and 56%) of the synaptic currents to a similar extent in both species.

contrast to pyramidal cells, the decay time course of mIPSCs in interneurons was significantly ($P < 0.05$, Mann–Whitney *U*-test) faster (rat, $\tau_w = 10.9 \pm 1.2$ ms, $n = 7$; mouse, $\tau_w = 19.6 \pm 2.6$ ms, $n = 5$), and the peak conductance was larger in rats than in mice (rat, 668 ± 49 pS; mouse, 510 ± 32 pS). Despite the differences in the amplitude and the decay kinetics of synaptic currents in CA1 interneurons, the effect of zolpidem was indistinguishable between the different species both in interneurons and in CA1 pyramidal cells (see below). Therefore, in the remaining cell types, postsynaptic $GABA_A$ receptor occupancy was only examined in mouse neurons.

Bath application of zolpidem (10 μM) significantly increased both the peak conductance (39 ± 11%, $P < 0.001$, *t*-test) and the decay time constant (52 ± 9%, $P < 0.001$) of mIPSCs recorded in mouse CA1 pyramidal cells (Fig. 1 and Table 1). A similar change in mIPSC amplitudes and kinetics was detected in rat neurons of the same type; a 35 ± 6% increase in the conductance and 73 ± 7% prolongation of the decay was observed ($P < 0.001$, Fig. 1D).

Hippocampal CA1 interneuron types were identified after the development of the biocytin labelling and comprised striatum oriens-lacunosum-molecurare cells ($n = 3$), horizontal and radial trilaminar cells ($n = 4$), or interneurons projecting to strata radiatum and lacunosum-moleculare ($n = 2$, Freund & Buzsáki, 1996; Hájos & Mody, 1997). As each interneuron type comprised a relatively small number of cells, we pooled all the interneurons for the description of the zolpidem effect on mIPSCs in these cells. Much like in principal cells, 10 μM zolpidem significantly ($P < 0.001$, Table 1) enhanced both the amplitude and decay of mIPSCs in mice (42 ± 4% and 61 ± 5%, respectively) and in rats (40 ± 5% and 84 ± 10%, respectively). In contrast to principal cells, however, the shift to the right of the cumulative distributions of mIPSC conductances was not always parallel (data not shown). In three out of five mouse interneurons, 20–40% of the events, in particular those with small amplitudes, showed no increase in their amplitudes following zolpidem application, but the prolongation of the decay of these

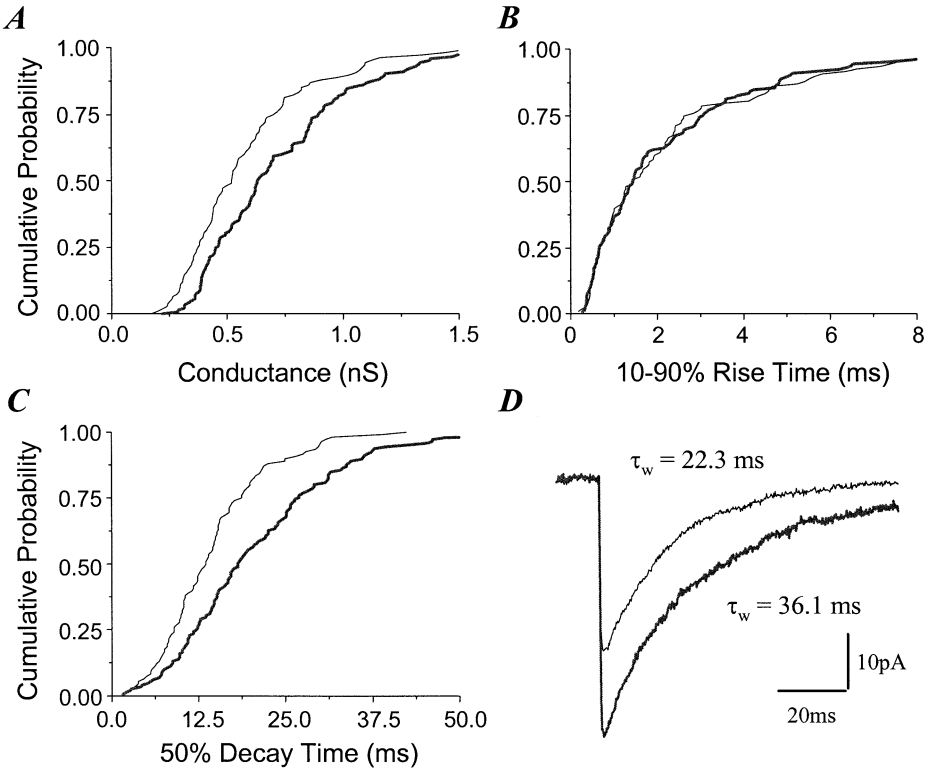

FIG. 2. Effects of 10 μM zolpidem on mIPSC properties recorded in a mouse dentate granule cell. Cumulative probability plots of mIPSC conductances (A), 10–90% rise times (B) and 50% decay times (C) before (thin lines) and after (thick lines) the application of zolpidem. The distributions of mIPSC conductances and 50% decay times in zolpidem are significantly different from those in the control (K–S test; $P < 0.001$), but no significant change was observed in the rise time distributions (K–S test, $P > 0.05$). (D) Averaged current waveforms in control (thin trace) and 10 μM zolpidem (thick trace) show that zolpidem increased both the peak amplitude and the decay time course of the synaptic currents. The decay phase of averaged mIPSCs can be well described by the sum of two exponential functions in the control [$\tau_1 = 16.5$ ms (74%), $\tau_2 = 38.8$ ms, $\tau_W = 22.3$ ms] and in zolpidem [$\tau_1 = 13.9$ ms (36%), $\tau_2 = 48.6$ ms, $\tau_W = 36.1$ ms].

mIPSCs was similar to that seen for events displaying a large amplitude enhancement (> 40%). These three cells did not belong to a single anatomical category of interneurons. A similar enhancement of mIPSC amplitudes has recently been described in cerebellar molecular layer interneurons (Nusser *et al.*, 1997). Accordingly, a different degree of $GABA_A$ receptor occupancy is predicted at distinct synapses of some hippocampal interneurons. More detailed investigations will be necessary to identify the precise origin of the distinct IPSCs.

Several previous studies examined the effect of zolpidem on mIPSCs of dentate granule cells at physiological temperature (33–36 °C) and reported the prolongation of decay times without a change in the amplitudes of the events (De Koninck & Mody, 1994; Hollrigel & Soltesz, 1997). However, as zolpidem should only be used to probe $GABA_A$ receptor occupancy at room temperature (Perrais & Ropert, 1999), we have re-examined its effect on mIPSCs in mouse dentate granule cells at room temperature. As shown in Fig. 2, mIPSC conductances (mIPSGs) as well as their durations were significantly increased, as indicated by a rightward shift of the cumulative distributions. The 10–90% rise times of mIPSCs remained constant (Fig. 2B). The significant enhancement of mIPSC amplitudes ($41 \pm 9\%$, $P < 0.001$) is consistent with an incomplete occupancy of the receptors in principal cells of the hippocampal formation.

Enhancement of mIPSC amplitudes by zolpidem is cell type specific in the neocortex

As previously shown, zolpidem increased the amplitude of mIPSCs in layer V pyramidal cells of young rats (Perrais & Ropert, 1999). We have confirmed this result in layer V pyramidal cells of mice. Following the application of zolpidem, the conductance of the synaptic currents increased from 581 ± 51 to 735 ± 74 pS, and their duration was also prolonged from 14.3 ± 1.9 to 23.9 ± 2.6 ms ($P < 0.001$, Table 1), an effect similar to that seen in layer V pyramidal cells of rats (Perrais & Ropert, 1999). Recordings were also obtained from GABAergic interneurons located in layer V of the neocortex. As the anatomical identification of cortical interneuron types was not performed for every cell, we pooled the layer V interneurons and presented the effects of zolpidem on the whole population. Compared with the effect of zolpidem on mIPSCs in layer V pyramidal cells, the mIPSG increase was somewhat larger ($38 \pm 3\%$ versus $26 \pm 4\%$), but the decay was less prolonged ($51 \pm 9\%$ versus $69 \pm 9\%$) in layer V interneurons.

To investigate whether zolpidem has a similar effect on mIPSCs recorded from neurons in supragranular layers, we obtained whole-cell voltage-clamp recordings from pyramidal cells and GABAergic interneurons in layer II/III. Application of 10–20 μM zolpidem had no significant effect on the peak conductance of the synaptic events recorded in layer II/III pyramidal cells ($102 \pm 4\%$ of control, $P > 0.4$). However, $GABA_A$ receptors underlying mIPSCs in layer II/III pyramidal cells were benzodiazepine sensitive, as 10–20 μM zolpidem significantly prolonged their decay kinetics ($56 \pm 6\%$, $P < 0.001$, Fig. 3). Figure 3 shows a representative cell. As seen on the cumulative distributions, zolpidem failed to change the conductance and 10–90% rise time of the events (K–S test, $P > 0.05$, Fig. 3A and B), but significantly prolonged their duration (K–S test, $P < 0.001$, Fig. 3C). In contrast to pyramidal cells in layer II/III, interneurons in the same layers responded to the administration of zolpidem by an increase in mIPSG and by the prolongation of the decay time ($22 \pm 3\%$ and $67 \pm 17\%$, respectively, $P < 0.001$, Table 1). Our results demonstrate that in most cell types of the neocortex, postsynaptic $GABA_A$ receptors are not fully occupied by the synaptically released GABA, similar to most cells of the hippocampus. However, postsynaptic $GABA_A$ receptor saturation does occur in some cell types (e.g. layer II/III pyramidal cells), consistent with a cell type-specific variation in the degree of occupancy.

Different degrees of receptor occupancy at GABAergic synapses of olfactory bulb granule cells

In olfactory bulb granule cells, the 10–90% rise times of mIPSCs have a bimodal distribution with a modal separation at ~ 1 ms,

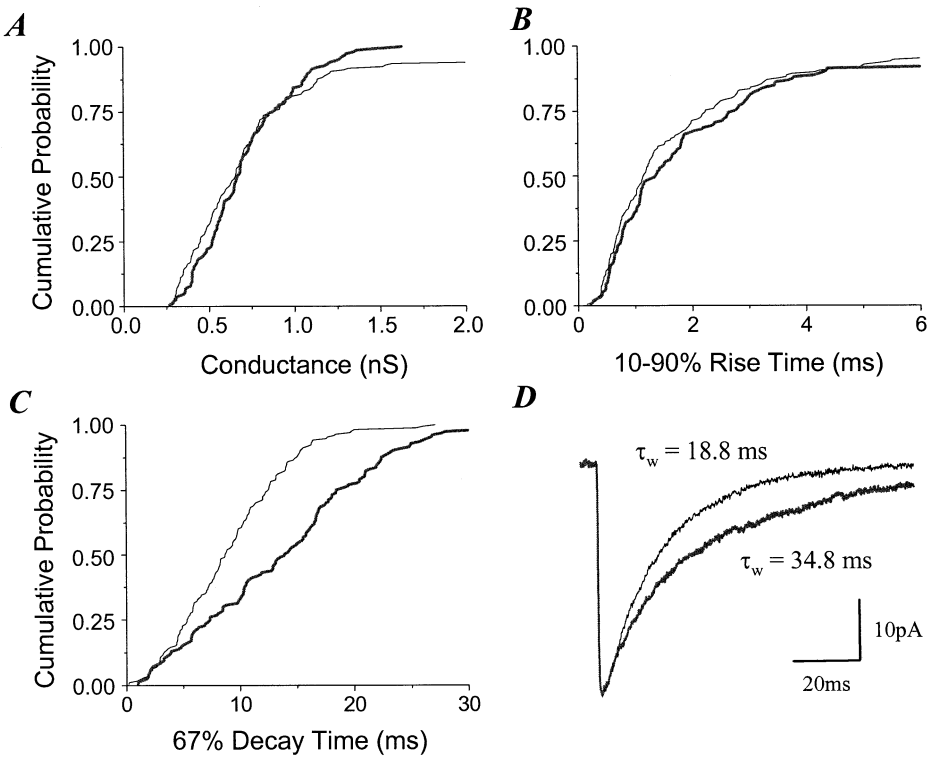

FIG. 3. Zolpidem increases the duration but not the peak conductance of mIPSCs in a mouse layer II/III pyramidal cell. Cumulative distributions of mIPSC conductances (A), 10–90% rise times (B) and 67% decay times (C) in control (thin lines) and in the presence of 20 μM zolpidem (thick lines). The distributions of mIPSC conductances and 10–90% rise times in zolpidem are not significantly different from those in the control (K–S test, $P > 0.05$). Zolpidem, however, significantly increased the decay time of mIPSCs, as indicated by a shift to the right on the cumulative distributions (K–S test, $P < 0.001$). (D) Superimposed averaged mIPSCs before (thin trace) and after the application of zolpidem (thick trace) demonstrate no change in the peak amplitude of the synaptic currents. In both cases, the decay was best fitted with the sum of two exponentials [control, $\tau_1 = 11.7$ ms (43%), $\tau_2 = 24.2$ ms, $\tau_W = 18.8$ ms; zolpidem, $\tau_1 = 10.8$ ms (37%), $\tau_2 = 49$ ms, $\tau_W = 34.9$ ms].

suggesting two distinct populations of events (Nusser *et al.*, 1999). Miniature IPSCs with 10–90% rise times faster than 1 ms ($mIPSC_{FR}$, 0.63 ± 0.07 ms) have three times faster rise times and almost twofold larger peak conductances (Table 1) than those with rise times slower than 1 ms ($mIPSC_{SR}$, 2.1 ± 0.35 ms). However, the weighted decay time constant of the slow rising mIPSCs ($\tau_w = 42.9 \pm 4.6$ ms) was similar to that of the $mIPSC_{FR}$ ($\tau_w = 45.9 \pm 6.5$ ms), indicating that dendritic filtering alone cannot be responsible for the differences between $mIPSC_{FR}$ and $mIPSC_{SR}$. The two types of event must have different kinetics at their site of generation, consistent with the possibility of originating from two functionally distinct types of synapses.

To examine whether postsynaptic $GABA_A$ receptor occupancy is similar at synapses generating these two types of synaptic current, we examined the effect of zolpidem (10–20 μM) on mIPSCs recorded from olfactory bulb granule cells at room temperature. $GABA_A$ receptors at synapses generating both $mIPSC_{FR}$ and $mIPSC_{SR}$ showed benzodiazepine sensitivity, as the decay of the synaptic currents was significantly prolonged after the bath application of zolpidem ($61 \pm 5\%$ and $76 \pm 16\%$, respectively, Fig. 4 and Table 1). In contrast, zolpidem significantly ($P < 0.02$) increased the amplitude of only the fast rising currents ($117 \pm 4\%$ of control, Fig. 4 and Table 1), but not that of the $mIPSC_{SR}$ ($102 \pm 4\%$ of control, Fig. 4 and Table 1). This result shows that the postsynaptic receptor occupancy could vary at different synapses on a given cell, in agreement with the result of previous studies in cerebellar neurons (Auger & Marty, 1997; Nusser *et al.*, 1997).

Discussion

Cell type-dependent variability in postsynaptic $GABA_A$ receptor occupancy

The effect of 10–20 μM zolpidem on the peak amplitude of mIPSCs was cell type specific, regardless of the subunit composition of the receptors. Zolpidem displaces Ro15-1788 from α1β3γ2, α2β3γ2, α3β3γ2 and α5β3γ2 $GABA_A$ receptors with K_i values of 20, 450, 400 and 15 000 nM, respectively (Pritchett & Seeburg, 1990), but we eliminated the impact of various subunits on zolpidem affinity by using 10–20 μM zolpidem, which should exert a similar effect on most BZ-sensitive $GABA_A$ receptor assemblies. Consequently, zolpidem increased the duration of all mIPSCs to a similar extent in every cell type studied. Thus, the cell type- and synapse-specific modulation of mIPSC peak conductances by zolpidem should reflect a varying degree of occupancy rather than differences in the zolpidem sensitivity of $GABA_A$ receptors. If 10–20 μM zolpidem increases postsynaptic receptor occupancy to near 1.0 following the synaptic release of GABA, the initial occupancy must vary between 0.7 and 0.8 at synapses with incomplete occupancy, and should be ~1.0 in layer II/III pyramidal cells. With a maximal open probability ($P_{o,max}$; as defined by Silver *et al.*, 1996) of $GABA_A$ receptors of 0.6–0.8 (Jones & Westbrook, 1997; Perrais & Ropert, 1999), the lower limit of the open probability ($P_o = P_{o,max} \times$ occupancy) is 0.42 and the upper limit is 0.8. If 10–20 μM zolpidem did not cause full occupancy, the lower limit of $P_o = 0.42$ is obviously an overestimate. As neither $P_{o,max}$ nor $GABA_A$ receptor occupancy depends on temperature (Perrais & Ropert, 1999), the values obtained at room temperature should hold true at physiological temperatures as well.

Occupancy is mainly determined by the postsynaptic target cell

Postsynaptic $GABA_A$ receptor saturation (full occupancy) occurs at synapses generating mIPSCs in layer II/III pyramidal neurons. In other cells of the same cortical layer, i.e. in GABAergic interneurons, the miniature inhibitory postsynaptic conductances (mIPSG) were enhanced by zolpidem, consistent with an incomplete occupancy. Yet, both pyramidal cells and interneurons of this layer are likely to be innervated by the same local inhibitory cells (Somogyi *et al.*, 1983; Kisvarday *et al.*, 1993; Thomson *et al.*, 1996; Tamas *et al.*, 1997,

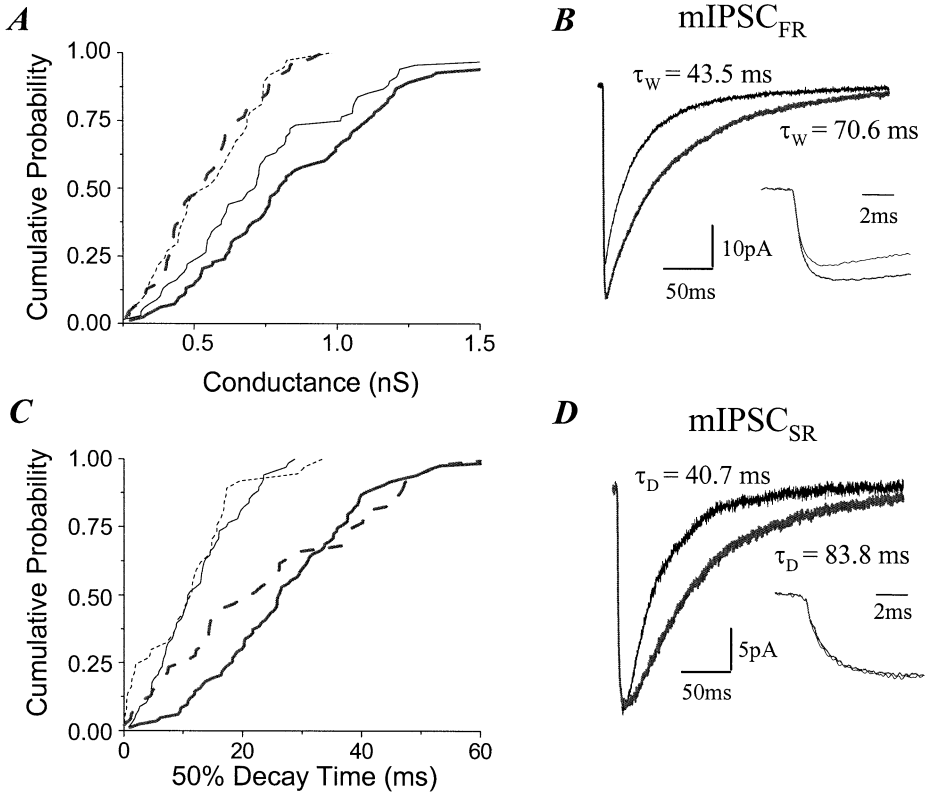

FIG. 4. The effect of zolpidem on mIPSCs is synapse specific in olfactory bulb granule cells. Miniature synaptic currents are grouped according to their rise times. mIPSCs with less than 1 ms 10–90% rise time comprise the fast rising currents ($mIPSC_{FR}$) while slow rising mIPSCs ($mIPSC_{SR}$) include those with slower rise times. (A) Cumulative distributions of mIPSC conductances demonstrate that the peak conductance of the $mIPSC_{SR}$ (thin dotted line in control) did not change significantly ($P > 0.1$, K–S test) after zolpidem application (thick dashed line), but that of $mIPSC_{FR}$ is increased (control, thin line; zolpidem, thick line; $P < 0.1$, K–S test). (B) Superimposed averaged $mIPSC_{FR}$ in control and in 10 μM zolpidem show that zolpidem increased both the peak amplitude and the decay time course of the synaptic currents. The decay phase of averaged $mIPSC_{FR}$ is best described by the sum of two exponential functions both in control [$\tau_1 = 26.7$ ms (79%), $\tau_2 = 106$ ms, $\tau_W = 43.5$ ms] and zolpidem [$\tau_1 = 35.8$ ms (45%), $\tau_2 = 107$ ms, $\tau_W = 70.6$ ms]. (C) Cumulative probability plots of the 50% decay times of $mIPSC_{FR}$ and $mIPSC_{SR}$ demonstrate an increase in the decay time of both slow and fast rising mIPSCs after zolpidem application (control $mIPSC_{SR}$, thin dotted line; control $mIPSC_{FR}$, thin line; zolpidem $mIPSC_{SR}$, thick dashed line; zolpidem $mIPSC_{FR}$, thick line). (D) Averaged slow rising mIPSCs show that only the decay time course of these events is prolonged by zolpidem without effecting the peak amplitude. The decay of the slow rising synaptic currents is well described by a single exponential function with time constants of 40.7 ms in control and 83.8 ms in zolpidem. (B and D) Insets show the rising phase of the synaptic currents on an expanded time scale.

TABLE 1. Effect of 10–20 μM zolpidem on mIPSCs recorded in eight different cell types of three brain regions of mice and rats

				Conductance (pS)			CV			τ_w (ms)		
Cell type	*n*	Age (days)	Species	Control	Zolpidem	Z/C ratio (%)	Control	Zolpidem	Z/C ratio (%)	Control	Zolpidem	Z/C ratio (%)
Olfactory GC $IPSC_{FR}$	5	20.4 ± 1.3	Mouse	1018 ± 189	1176 ± 4	117 ± 4*	0.51 ± 0.03	0.47 ± 0.02	93 ± 7	45.9 ± 6.5	74.4 ± 11.1	161 ± 5*
Olfactory GC $IPSC_{SR}$	5	20.4 ± 1.3	Mouse	577 ± 83	595 ± 95	102 ± 4	0.39 ± 0.03	0.49 ± 0.07	126 ± 21	42.9 ± 4.6	76.9 ± 12.3	176 ± 16*
Layer II/III PC	8	21.4 ± 3.3	Mouse	606 ± 58	606 ± 46	102 ± 4	0.42 ± 0.03	0.42 ± 0.02	101 ± 6	21.2 ± 1.4	32.9 ± 2.1	156 ± 6*
Layer II/III IN	6	32.4 ± 0.7	Mouse	513 ± 92	633 ± 127	122 ± 3*	0.45 ± 0.04	0.5 ± 0.04	114 ± 5	19.6 ± 2.4	33.3 ± 5.6	167 ± 17*
Layer V PC	6	24.2 ± 3.0	Mouse	581 ± 51	735 ± 74	126 ± 4*	0.44 ± 0.04	0.47 ± 0.03	110 ± 7	14.3 ± 1.9	23.0 ± 2.6	169 ± 9*
Layer V IN	6	37.7 ± 0.8	Mouse	566 ± 41	779 ± 48	138 ± 3*	0.52 ± 0.03	0.54 ± 0.04	104 ± 3	17.0 ± 2.0	25.6 ± 3.0	151 ± 9*
Dentate GC	6	29.7 ± 2.8	Mouse	499 ± 34	691 ± 32	141 ± 9*	0.48 ± 0.03	0.55 ± 0.05	114 ± 10	20.3 ± 1.5	33.2 ± 3.0	163 ± 6*
CA1 PC	6	33.7 ± 2.6	Mouse	508 ± 31	693 ± 19	139 ± 11*	0.45 ± 0.01	0.48 ± 0.01	107 ± 4	20.1 ± 1.7	29.9 ± 1.3	152 ± 9*
CA1 IN	5	24.4 ± 1.1	Mouse	510 ± 32	719 ± 34	142 ± 4*	0.5 ± 0.04	0.6 ± 0.05	120 ± 6	19.6 ± 2.6	31.2 ± 4.1	161 ± 5*
CA1 PC	5	17.6 ± 0.2	Rat	614 ± 35	834 ± 78	135 ± 6*	0.42 ± 0.02	0.47 ± 0.02	112 ± 6	17.8 ± 1.8	30.6 ± 2.9	173 ± 7*
CA1 IN	7	17.8 ± 0.3	Rat	668 ± 49	940 ± 79	140 ± 5*	0.5 ± 0.01	0.54 ± 0.03	109 ± 4	10.9 ± 1.2	19.9 ± 2.1	184 ± 10*

Conductance values are obtained as the mean of the mIPSC conductance distributions. The coefficient of variation (CV) is calculated by dividing the SD of the mIPSC conductance distribution with its mean. The weighted decay time constant (τ_w) is calculated as the weighted average of the time constants of fast and slow exponential components fitted to the averaged synaptic currents. Data are given as mean ± SEM. *n* indicates the number of recorded cells. The ratio Z/C (%) denotes the mean ± SEM of the corresponding parameters in zolpidem relative to the control values. The age of the animals is given in days. Olfactory GC, granule cells of the olfactory bulb; PC, pyramidal cell; IN, interneuron; dentate GC, granule cell of the dentate gyrus.

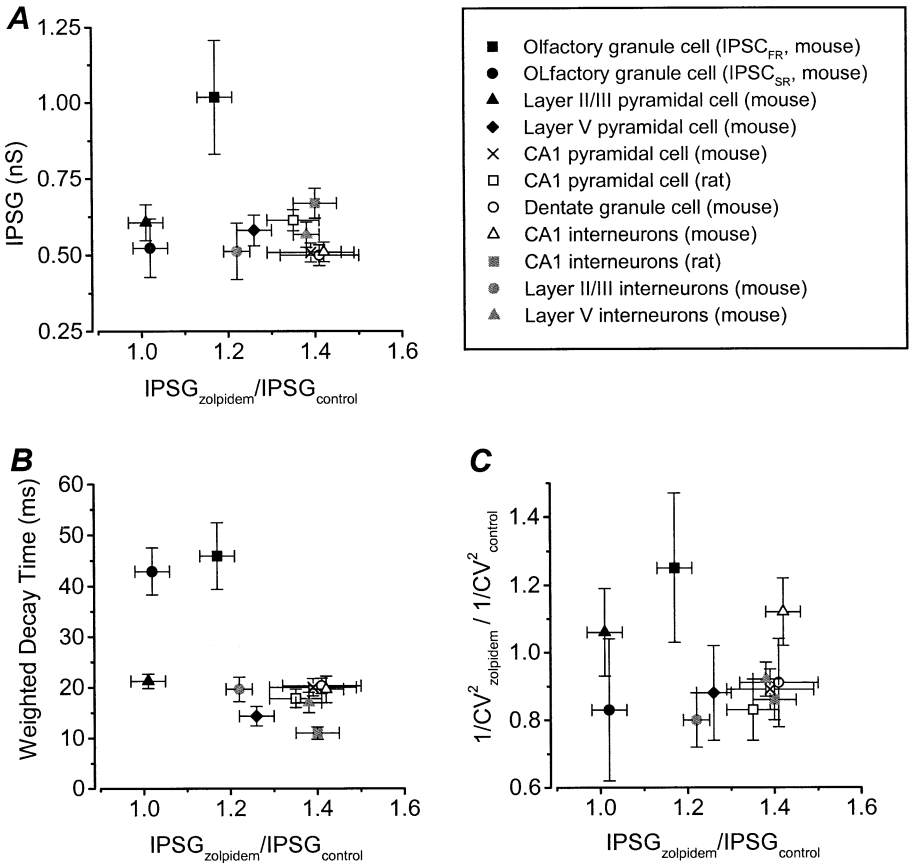

FIG. 5. The degree of synaptic $GABA_A$ receptor occupancy shows no significant correlation with mIPSC conductances (A) or decay times (B) in different cell types. The $mIPSG_{zolpidem}$: $mIPSG_{control}$ ratio indicates the degree of occupancy under control conditions, such that a larger ratio corresponds to a smaller postsynaptic receptor occupancy. (C) The $1/CV^2_{zolpidem}$: $1/CV^2_{control}$ ratio is a measure of the change in the variability of the peak amplitudes after zolpidem application. This ratio is independent of the initial occupancy of the postsynaptic receptors, suggesting that the main variability in the peak amplitude does not originate from the variation of the transmitter concentration in the cleft.

1998). Thus, $GABA_A$ receptor occupancy appears to be predominantly determined by the identity of the postsynaptic target cell. In most cell types, zolpidem increased the peak conductance of mIPSCs, indicating an incomplete postsynaptic receptor occupancy (Frerking *et al*., 1995; Defazio & Hablitz, 1998; Perrais & Ropert, 1999). The parallel shift in the cumulative mIPSG distributions caused by zolpidem in most cells indicates a similar degree of receptor occupancy at all synapses responsible for generating mIPSCs, and is also consistent with receptor occupancy being controlled by some postsynaptic mechanism. In dentate granule cells mIPSCs originate from perisomatic synapses (Soltesz *et al*., 1995), and at least three interneuron types form synapses in this region (Freund & Buzsáki, 1996) having $GABA_A$ receptors with different subunit compositions depending on the identity of the presynaptic neuron (Nusser *et al*., 1996). Yet, according to the parallel shift in mIPSG distributions in zolpidem, at each mIPSC-generating synapse receptors have a similar degree of occupancy regardless of the origin of the presynaptic terminals.

In most cells the degree of occupancy seems to be determined by the postsynaptic target cell, but a synapse-specific variation in the occupancy within a single cell might mean that in such cells other (e.g. presynaptic) factors also contribute to the control of postsynaptic receptor occupancy. Two previous studies have suggested that at distinct synapses of a single cell (cerebellar stellate cells), postsynaptic $GABA_A$ receptor occupancy could vary (Auger & Marty, 1997; Nusser *et al*., 1997). In three out of five GABAergic interneurons of the hippocampal CA1 area, zolpidem caused a non-parallel shift in the cumulative distribution of mIPSGs. Furthermore, of the two distinct populations of zolpidem-sensitive mIPSCs recorded in olfactory bulb granule cells, the drug significantly enhanced the amplitude of only one type of event ($mIPSC_{FR}$).

Variation in postsynaptic $GABA_A$ receptor occupancy is not solely the consequence of variation in the number of postsynaptic receptors

The following three possibilities can be envisaged to explain differences between synapses with complete versus incomplete receptor occupancy: (i) different numbers of receptors are present at synapses of distinct sizes; (ii) synapses contain $GABA_A$ receptors with distinct GABA affinities; and (iii) different concentrations of GABA are released into the synaptic cleft.

In cerebellar stellate cells, the P_o of $GABA_A$ receptors at large synapses, containing many $GABA_A$ receptors, is lower than that at small synapses with few receptors (Auger & Marty, 1997; Nusser *et al*., 1997). Accordingly, $GABA_A$ receptor occupancy may be determined by the number of postsynaptic receptors. At small synapses with only a few $GABA_A$ receptors (<80), the receptors should be saturated by the released GABA, whereas at large synapses the hundreds of postsynaptic receptors are not fully occupied. It follows that large-amplitude mIPSCs should be increased by zolpidem more than those with small amplitudes. Inconsistent with the possibility that the number of postsynaptic receptors alone is responsible for determining receptor occupancy, we found no significant correlation between the peak conductance of mIPSCs and the initial occupancy of the receptors (reflected by the $IPSG_{zolpidem}$: $IPSG_{control}$ ratio; Fig. 5A) in eight different cell types of three brain regions.

Variation in postsynaptic $GABA_A$ receptor occupancy is not solely the consequence of receptors with different GABA affinity

The variation in the degree of occupancy between cells and synapses may be the consequence of different $GABA_A$ receptors with distinct

affinity for GABA. The microscopic binding rate is inversely proportional to the unbinding rate at $GABA_A$ receptors (Jones *et al*., 1998). As the agonist unbinding rate strongly influences deactivation and consequently synaptic current decay, lower affinity receptors should produce more rapidly decaying synaptic currents (Jones *et al*., 1998). If the variation in synaptic GABA receptor occupancy results from the presence of receptors with different affinities for GABA, IPSCs with fast decays (i.e. those generated by low-affinity receptors) should be more strongly potentiated by zolpidem. Yet our data show no correlation between the decay of the synaptic currents and the initial receptor occupancy (Fig. 5B). Provided that the binding and unbinding rates are indeed inversely proportional (Jones *et al*., 1998) for every $GABA_A$ receptor subtype, our results suggest that the varying degrees of occupancy seen at different synapses are not simply a consequence of receptors with diverse GABA affinities.

Variability in the transmitter concentration may be the major contributor to the variation of postsynaptic receptor occupancy between cells and synapses

The estimates of peak GABA concentrations in the synaptic cleft range from 0.3 to 3 mM (Maconochie *et al*., 1994; Jones & Westbrook, 1995; Mozrzymas *et al*., 1999; Perrais & Ropert, 1999). This large range in the estimates may stem from studying diverse synapses. Morphological data also predict large differences in the GABA concentration at synapses. The volume of GABAergic synapses varies by 20-fold in cerebellar interneurons (Nusser *et al*., 1997) and by over 10-fold in dentate gyrus granule cells (Nusser *et al*., 1998). Because our data do not support the dependence of occupancy on GABA receptor number or affinity, the variation in transmitter concentration in the cleft could be responsible for the variation in the postsynaptic receptor occupancy. Clearly, future experiments are required to directly test this hypothesis, including the manner in which the postsynaptic cell matches the cleft GABA concentration to the number and type of receptors to produce a relatively constant occupancy across different synapses.

The variation in the GABA concentration in the cleft was also proposed to underlie the amplitude variability of postsynaptic responses (Frerking *et al*., 1995). Other studies identified differences in the postsynaptic receptor number as determining the variability in quantal amplitude (Edwards *et al*., 1990; De Koninck & Mody, 1994; Nusser *et al*., 1997, 1998; Lim *et al*., 1999). If the variance in quantal amplitude within a cell solely results from the trial-to-trial fluctuation of the transmitter concentration at synapses with unsaturated receptors, an increase in occupancy, e.g. caused by zolpidem, should reduce quantal variance. We monitored (Fig. 5C) possible changes in the variability of the peak amplitudes after zolpidem application (given by the ratio $1/CV^2_{zolpidem} : 1/CV^2_{control}$) as a function of the degree of occupancy (described by the ratio $IPSG_{zolpidem} : IPSG_{control}$). According to the lack of a correlation (Fig. 5C), variation in cleft transmitter concentration cannot be the major determinant of the quantal variance. Rather than the trial-to-trial fluctuation of GABA concentration in the synaptic cleft, the 20–30-fold variability in postsynaptic $GABA_A$ receptor number shown to exist in cerebellar stellate cells and dentate gyrus granule cells (Nusser *et al*., 1997, 1998) is more likely to produce the amplitude variance of IPSCs.

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Abbreviations

ACSF, artificial cerebrospinal fluid; CV, coefficient of variation; GABA, γ-aminobutyric acid; $GABA_A$ receptor, type A γ-aminobutyric acid receptor; IPSCs, inhibitory postsynaptic currents; mIPSCs, miniature inhibitory postsynaptic currents; $mIPSC_{FR}$, fast rising miniature inhibitory postsynaptic current; $mIPSC_{SR}$, slow rising miniature inhibitory postsynaptic current; mIPSGs, miniature inhibitory postsynaptic conductances; P_o, channel open probability; $P_{o,max}$, maximal channel open probability; PB, phosphate buffer; TTX, tetrodotoxin; τ_w, weighted decay time constant.

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