GABA transient sets the susceptibility of mIPSCs to modulation by benzodiazepine receptor agonists in rat hippocampal neurons

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Benzodiazepines (BDZs) are known to increase the amplitude and duration of IPSCs. Moreover, at low [GABA], BDZs strongly enhance GABAergic currents suggesting the up-regulation of agonist binding while their action on gating remains a matter of debate. In the present study we have examined the impact of flurazepam and zolpidem on mIPSCs by investigating their effects on $GABA_A$R binding and gating and by considering dynamic conditions of synaptic receptor activation. Flurazepam and zolpidem enhanced the amplitude and prolonged decay of mIPSCs. Both compounds strongly enhanced responses to low [GABA] but, surprisingly, decreased the currents evoked by saturating or half-saturating [GABA]. Analysis of current responses to ultrafast GABA applications indicated that these compounds enhanced binding and desensitization of $GABA_A$ receptors. Flurazepam and zolpidem markedly prolonged deactivation of responses to low [GABA] but had almost no effect on deactivation at saturating or half-saturating [GABA]. Moreover, at low [GABA], flurazepam enhanced desensitization–deactivation coupling but zolpidem did not. Recordings of responses to half-saturating [GABA] applications revealed that appropriate timing of agonist exposure was sufficient to reproduce either a decrease or enhancement of currents by flurazepam or zolpidem. Recordings of currents mediated by recombinant ('synaptic') $\alpha1\beta2\gamma2$ receptors reproduced all major findings observed for neuronal $GABA_A$Rs. We conclude that an extremely brief agonist transient renders IPSCs particularly sensitive to the up-regulation of agonist binding by BDZs.

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GABA (γ-aminobutyric acid) is the major inhibitory neurotransmitter in the adult mammalian central nervous system. To date, as many as 20 subunits of $GABA_A$ receptors (γ-aminobutyric acid receptor type A) (α1–6, β1–4, γ1–3, δ, ρ1–3, ε, π and θ) have been cloned (Cherubini & Conti, 2001; Fritschy & Brunig, 2003) suggesting an overwhelming heterogeneity. However, most common $GABA_A$Rs consist of two α, two β and one γ or δ subunit (Whiting, 2003; Wafford, 2005).

Benzodiazepine (BDZ) receptor agonists are known as positive modulators of specific $GABA_A$ receptors ($GABA_A$Rs) (Rudolph & Mohler, 2004; Wafford, 2005; Rudolph & Mohler, 2006). BDZs were commonly found to enhance the amplitude and to prolong GABAergic IPSCs (inhibitory postsynaptic currents) (Frerking *et al.* 1995; Nusser *et al.* 1997; Perrais & Ropert, 1999; Hajos *et al.* 2000; Perrais & Ropert, 2000). Several lines of evidence indicate that BDZs up-regulate the binding affinity of $GABA_A$Rs. A compelling indication for this mechanism is a strong BDZ-induced enhancement of amplitude and onset rate of currents evoked by non-saturating [GABA] but these effects tend to disappear at saturating [GABA] (Lavoie & Twyman, 1996; Krampfl *et al.* 1998). BDZs do not clearly affect the $GABA_A$R single channel lifetimes (Twyman *et al.* 1989; Rogers *et al.* 1994). These findings, taken altogether, suggest that BDZs enhance binding affinity while their effect on $GABA_A$R gating appears minor. However, Rusch & Forman (2005) as well as Downing *et al.* (2005) have proposed a novel mechanism for $GABA_A$R modulation by BDZs. They considered a spontaneously active $GABA_A$R mutant and reported that BDZs might effectively modulate the $GABA_A$R gating. However, it was not clear to what extent these observations applied to the native $GABA_A$Rs in which coupling between binding and gating has not been altered by mutations. More recently, Campo-Soria *et al.* (2006) have reported that in oocytes expressing

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ultrahigh levels of wild type $GABA_A$ receptors, a high concentration (1 μM) of diazepam induced a small but detectable GABAergic current and proposed that this BDZ might act on $GABA_A$Rs by affecting the opening transitions. In a recent study, we have reported that flurazepam and zolpidem affected both binding and gating of $\alpha1\beta2\gamma2$ receptors (Mercik *et al.* 2007). The effect on receptor gating was deduced from a BDZ-induced decrease in amplitude and a moderate modulation of the time course of currents elicited by saturating [GABA]. These observations appeared peculiar as they were substantially different from what is commonly observed for synaptic currents. It is thus interesting to investigate whether the effects of flurazepam and zolpidem observed for $\alpha1\beta2\gamma2$ receptors can be reproduced in neurons. In particular, it is appealing to compare the action of these drugs on currents evoked by exogenous GABA and on mIPSCs (miniature inhibitory postsynaptic currents). For this purpose, the effect of flurazepam and zolpidem has been examined on mIPSCs and on current responses to ultrafast GABA applications recorded from rat cultured hippocampal neurons. We found that flurazepam and zolpidem modulated both binding and gating of neuronal receptors. However, BDZ effects on amplitudes of mIPSCs and on responses to saturating [GABA] showed qualitative differences. To explore this discrepancy, we have modelled the synaptic currents by responses to short applications of non-saturating [GABA] and concluded that extreme non-equilibrium conditions, dictated by a fast agonist transient, render mIPSCs particularly susceptible to modulation by BDZs.

Methods

Neuronal primary cell culture

Primary cell culture was prepared as already described (Andjus *et al.* 1997). Briefly, P2–P4 Wistar rats were killed by decapitation. This procedure is in agreement with the Polish Animal Welfare Act and has been approved by the Local Ethical Commission. Hippocampi were dissected, sliced, treated with trypsin, mechanically dissociated and centrifuged twice at 40 *g*, plated in Petri dishes and cultured. Experiments were performed on cells between 10 and 15 days in culture.

Expression of recombinant receptors

The recombinant $\alpha1\beta2\gamma2$S $GABA_A$ receptors were expressed in the HEK293 cell line (human embryonic kidney cells). HEK293 cells were cultured in Dulbecco's modified Eagle's medium with Glutamax-I (Gibco BRL) supplemented with 5% fetal bovine serum (Gibco BRL), 100 U ml^{-1} penicillin G, and 100 μg ml^{-1} streptomycin in an incubator at 37°C with 5.0% CO_2. Transfection was performed using a standard calcium phosphate precipitation protocol (Chen & Okayama, 1987) with plasmids pGW1 ampicillin-resistant containing cDNA encoding for rat $\alpha1$, $\beta2$, $\gamma2$S or $GABA_A$ receptor. Plasmids were cotransfected at individual concentrations of 1 μg ml^{-1} together with the pCVMCD4 plasmid to encode the CD4 receptor. Transfected cells were identified by markers (beads) covered with anti-CD4 antibodies (Dynabeads M–450 CD4, Dynal Biotech ASA, Oslo, Norway).

Electrophysiological recordings

Currents were recorded either from neurons or from HEK293 cells in the outside-out mode of the patch-clamp technique using the Axopatch 200B amplifier (Molecular Device Corporation, Sunnyvale, CA, USA) at −70 mV. The intrapipette solution contained: (mM) 137 CsCl, 1 $CaCl_2$, 2 $MgCl_2$, 11 BAPTA (tetracaesium salt), 2 ATP and 10 Hepes, pH 7.2 with CsOH. The external solution was composed of (mM): 137 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 20 glucose and 10 Hepes, pH 7.2 with NaOH. The current signals were filtered at 10 kHz with a Butterworth filter, sampled at 50–100 kHz, using the analog-to-digital converter Digidata 1322A (Molecular Device Corporation) and stored on the computer hard disk. pCLAMP 9.2 (Molecular Device Corporation) software was used for acquisition and data analysis. Miniature synaptic currents were recorded in the whole-cell configuration of the patch clamp technique in the presence of 1 μM tetrodotoxin (TTX). Current responses to 1 μM GABA were barely detectable in the excised patch configuration and for this reason were recorded in the whole-cell mode using a multibarrel system (RSC-200, Bio-Logic, Grenoble, France; exchange time *ca* 8–15 ms). In most cases, responses to 3 μM GABA obtained from excised patches were too small to be reliably analysed and therefore most of the responses to this GABA concentration were collected in the whole-cell mode as in the case of 1 μM GABA.

Rapid drug application

GABA was applied to excised patches using the ultrafast perfusion system based on a piezoelectric-driven theta-glass application pipette (Jonas, 1995). The piezoelectric translator was from Physik Instrumente (preloaded HVPZT translator 80 μm, Waldbronn, Germany) and theta-glass tubing was from Hilgenberg (Malsfeld, Germany). The open tip recordings of the liquid junction potentials revealed that 10–90% exchange of solution occurred within 50–80 μs. A minimum duration of drug application was 0.8–1 ms (at shorter pulses, often oscillations appeared). In experiments in which

the effect of BDZ receptor agonists was examined, the tested modulator was present at the same concentrations in solutions supplied by both channels (wash and GABA-containing solution) of the theta-glass capillary. Before applying the agonist (in the presence or absence of BDZ) the patch was exposed to the washing solution for at least 2 min. Occasionally, in cells with high $GABA_AR$ expression, current responses to 3 μM GABA were studied in the excised patches using ultrafast perfusion. The current characteristics such as rise time and deactivation of responses recorded in the whole-cell and excised-patch modes were not clearly different. Due to extreme difficulty of experiments with ultrafast application system we have focused our study on testing the effects of flurazepam and zolpidem at micromolar concentrations that were close to saturation for both drugs (e.g. Tietz *et al.* 1999; Walters *et al.* 2000). For responses to low [GABA] (1–3 μM), 1 μM of flurazepam or zolpidem yielded a submaximal effect that reached saturation at 3 μM (zolpidem) or between 3 and 10 μM (flurazepam, data not shown).

Data analysis

The kinetics of the current rising phase was quantified as 10–90% rise time. Deactivation current was fitted with a sum of two exponential functions:

$$y(t) = A_1 \exp(-t/\tau_{\text{fast}}) + A_2 \exp(-t/\tau_{\text{slow}})$$

where A_1 and A_2 are the percentages while τ_{fast} and τ_{slow} are the time constants. For normalized currents, $A_1 + A_2 = 1$. The mean time constant was calculated as $\tau_{\text{mean}} = A_1\tau_{\text{fast}} + A_2\tau_{\text{slow}}$. The desensitization kinetics was described by a sum of one exponential function and a constant value representing the steady-state current. Recovery parameter R was defined as $R = (I_2 - I_3)/(I_1 - I_3)$, where I_1 the first peak amplitude, I_2 the second peak amplitude, and I_3 is the current immediately before the second pulse. The effect of flurazepam and zolpidem on IPSCs and on current responses was assessed from the comparison between control and test recordings from the same cell (or excised patch). For this reason all the results are presented as relative values normalized to the respective controls.

The kinetic modelling was performed using the ChannelLab 2.0 software (developed by S. Traynelis for Synaptosoft, Decatour, GA, USA). This software converted the kinetic model into a set of differential equations and solved them numerically assuming, as the initial condition, that at $t = 0$ (where t is time), no bound or open receptors were present. The solution of such equations predicted the time courses of occupancies of all the states included in the model. The current time course was modelled as the time evolution of the sum of open state occupancies.

Data are expressed as mean ± S.E.M. and Student's t test was used for the comparison of data.

All experiments were performed at room temperature (22–24°C).

Results

Flurazepam and zolpidem enhance amplitude and slow down the decaying phase of mIPSCs

Miniature IPSCs were recorded in the whole-cell configuration at −70 mV in the presence of 1 μM TTX (Fig. 1*A*). On average, in control conditions, mIPSC amplitude was -41.21 ± 2.22 pA ($n = 21$). The averaged mIPSC frequency in control conditions was 0.26 ± 0.02 Hz ($n = 21$) and it was not significantly affected by either flurazepam or zolpidem (data not shown). Addition of flurazepam resulted in a significant increase in the mean mIPSC amplitude for the entire range of considered flurazepam concentrations (1–10 μM, Fig. 1*B*) and in a clear shift in the cumulative amplitude distribution (Fig. 1*C*). With 1 μM flurazepam, the relative amplitude was 1.20 ± 0.06 ($n = 11$). Similarly, zolpidem at concentrations of 0.3–3 μM, significantly enhanced mean mIPSC amplitudes (Fig. 1*D* and *E*; at 1 μM zolpidem, the relative amplitude increase was 1.23 ± 0.07, $n = 6$).

The flurazepam-induced increase in mIPSC amplitude was accompanied by a change in the current kinetics. In control conditions, the decaying phase of mIPSCs could be well fitted with a sum of two exponentials ($\tau_{\text{fast}} = 11.83 \pm 1.06$ ms, $\tau_{\text{slow}} = 61.37 \pm 3.8$ ms, $A_{\text{slow}} = 0.46 \pm 0.03$, $n = 11$) and the mean decay time constant was $\tau_{\text{mean}} = 36.28 \pm 1.92$ ms. As shown in Fig. 2*B*, the value of τ_{mean} was increased by flurazepam in a dose-dependent manner. This effect was associated with a clear prolongation of the slower decay component (τ_{slow}, Fig. 2*C*) with no apparent effect on its percentage (A_{slow}, Fig. 2*D*). Zolpidem significantly prolonged the mean time constant of mIPSC decaying phase at concentrations of 1 and 3 μM (Fig. 2*F*) and this change was related to an increase in the percentage of the slow component with no effect on the slow time constant (Fig. 2*G* and *H*).

Flurazepam and zolpidem exert opposite effects on amplitudes of currents elicited by low and high [GABA]

Results presented in Figs 1 and 2 confirmed that in our model both flurazepam and zolpidem enhanced amplitudes and prolonged decay of mIPSCs, although the effects of these drugs showed some differences. To explore the mechanism underlying such a modulation, current responses to rapid GABA applications were measured. Control responses to various GABA concentrations were recorded (examples in Fig. 3*A*–*C* thin lines) and a dose–response relationship was constructed from values

of current amplitudes normalized to the amplitude of responses elicited by 10 mM GABA measured from the same patch (Fig. 3*D*, in control conditions, currents elicited by 10 mM GABA had the averaged amplitude of -789 ± 198 pA, $n = 17$). From these recordings we could estimate that the GABA concentration evoking a half-maximal amplitude was close to 100 μM (Fig. 3*D*). As expected, current responses to low [GABA] (1–3 μM) were strongly potentiated both by flurazepam and zolpidem but the effect of the latter was significantly larger at 1 μM GABA (Fig. 3*A* and *E*). However, when increasing [GABA] to 10 or 30 μM, the current enhancement was no longer present (Fig. 3*E*) and for 100 μM or 10 mM GABA both BDZ receptor agonists induced a significant current reduction (Fig. 3*B*, *C* and *E*). These data demonstrate that BDZ receptor agonists down modulate the responses of neuronal $GABA_A$Rs to saturating or half-saturating [GABA], similarly to what was observed for recombinant $\alpha 1\beta 2\gamma 2$ receptors (Mercik *et al.* 2007).

Flurazepam and zolpidem differentially affect the deactivation kinetics of currents elicited by low and high [GABA]

Recordings of synaptic currents confirmed that both flurazepam and zolpidem prolonged the decaying phase of mIPSCs (Fig. 2). Since GABA synaptic transient is very brief (Clements, 1996; Mozrzymas *et al.* 1999, 2003*b*; Overstreet *et al.* 2002; Mozrzymas, 2004) it is believed that mIPSC decay is largely determined by the deactivation process (time relaxation after agonist removal). It is thus interesting to check how flurazepam and zolpidem affect the deactivation kinetics of currents evoked by different GABA concentrations. For this purpose, currents were elicited by GABA pulse whose duration was sufficient for the current to reach its maximum (or plateau). For instance, at 1 μM GABA, a 2 s pulse was applied (Fig. 4*A*) and in these conditions, in 19 out of 28 cells, the deactivation kinetics was characterized by a biexponential

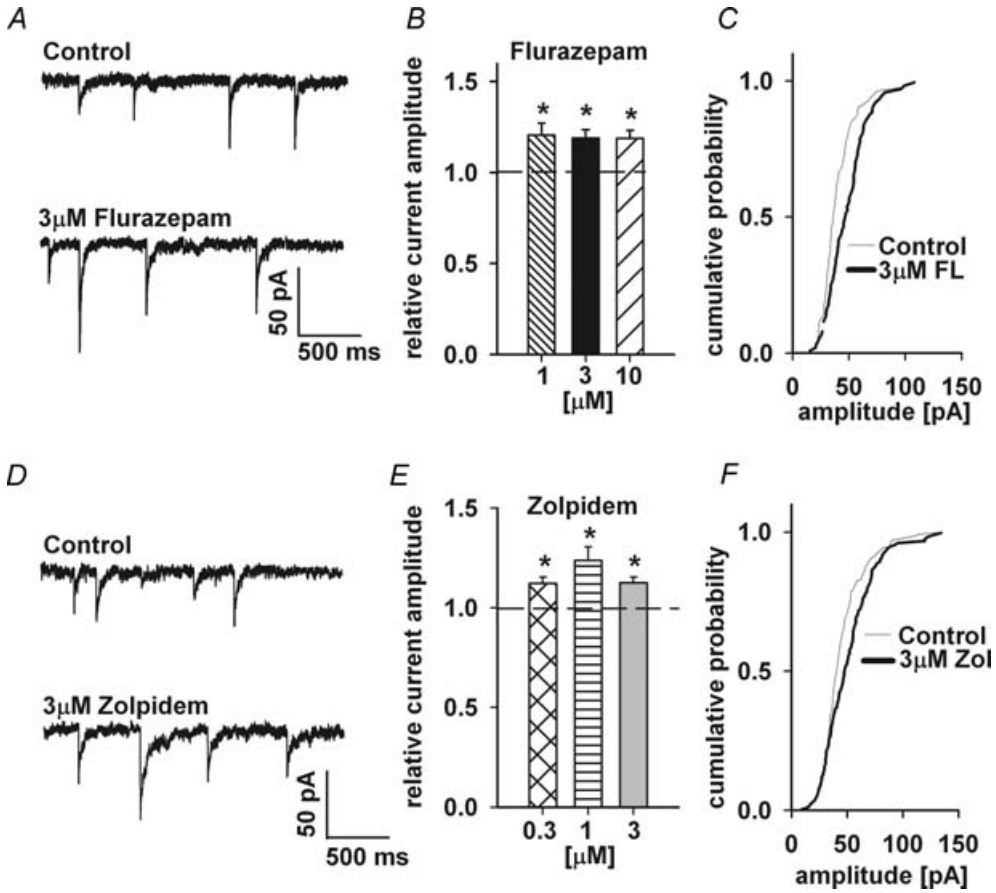

Figure 1. Flurazepam and zolpidem enhance the amplitude of mIPSCs
A, examples of mIPSCs recorded at −70 mV in control conditions (upper trace) and in the presence of 3 μM flurazepam (FL; lower trace). *B*, statistics of flurazepam effect on mIPSC amplitude. *C*, typical cumulative histogram for a record in control conditions (thin line) and in the presence of 3 μM flurazepam (thick line). *D*, examples of mIPSCs recorded at −70 mV in control conditions (upper trace) and in the presence of 3 μM zolpidem (Zol; lower trace). *E*, statistics of zolpidem effect on mIPSC amplitude. *F*, typical cumulative histograms for control conditions (thin line) and mIPSCs recordings in the presence of 3 μM zolpidem (thick line). Mean values were calculated from at least $n = 5$ cells. *Significant difference with respect to the control values.

time course ($\tau_{fast} = 191 \pm 21$ ms, $\tau_{slow} = 880 \pm 82$ ms, $A_{slow} = 0.24 \pm 0.03$, $n = 19$) and in 9 out of 28 cells, a single exponential fit was made ($\tau = 288 \pm 34$ ms, $n = 9$). The mean deactivation time constant (1 μM GABA, 2 s application) was: $\tau_{mean} = 328 \pm 28$ ms ($n = 28$). The absolute values of τ_{mean} for currents recorded using this experimental protocol showed a tendency to decrease with GABA concentration (Fig. 4*B*) that, as explained in detail in the next section, appears to be related to an increasing contribution of rapid desensitization at high [GABA]. Interestingly, both BDZ receptor agonists significantly slowed down the deactivation time course (for 2 s application of 1 μM GABA, relative $\tau_{mean} = 1.70 \pm 0.12$, $n = 5$, for 3 μM flurazepam; 1.88 ± 0.15, $n = 10$, for 3 μM zolpidem, $P < 0.05$, Fig. 4*A* and *C*). The prolongation of deactivation kinetics by these drugs resulted mainly from the enhancement of percentage of the slow component (data not shown). However, as shown in Fig. 4*C*, when increasing [GABA] the effect of BDZ receptor agonists on deactivation kinetics progressively decreased. For flurazepam (3 μM), the prolongation of deactivation was significant for GABA concentrations up to 10 μM and for zolpidem (3 μM) up to 30 μM GABA (Fig. 4*C*). Since current responses to high [GABA] may provide essential information on receptor gating, it is important to describe in more detail the deactivation process for currents elicited by saturating [GABA] (see also sections below). Decaying phases of currents evoked by short pulses (1–3 ms) of 10 mM GABA were analysed to describe the deactivation kinetics (alteration of application time within 1–3 ms did not affect the time course of current responses to 10 mM GABA). Decay of these currents could be well fitted with a sum of two exponentials ($\tau_{fast} = 2.92 \pm 0.87$ ms, $\tau_{slow} = 125 \pm 11.40$ ms, $A_{slow} = 0.26 \pm 0.03$, $n = 20$) and the weighted average deactivation time constant was $\tau_{mean} = 36.13 \pm 5.91$ ms ($n = 20$). Flurazepam, at concentration up to 3 μM, had no significant effect on deactivation kinetics (Fig. 4*D* and *E*). Interestingly, at

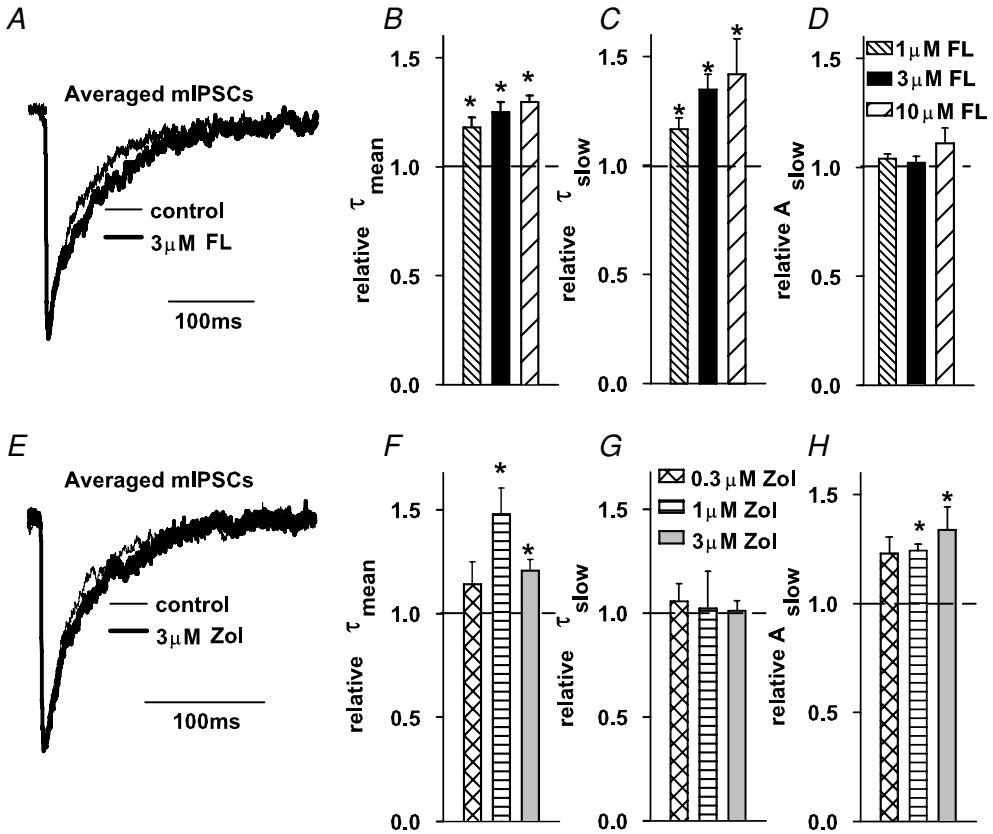

Figure 2. Flurazepam and zolpidem prolong the decaying phase of mIPSCs
A, typical averaged and superimposed mIPSCs recorded in control conditions (thin line) and in the presence of 3 μM flurazepam (thick line). *B*, statistics of the flurazepam effect on the mean decay time constant (τ_{mean}). Flurazepam prolongs the slow decay component (τ_{slow}, *C*) without affecting the percentage of this component (A_{slow}, *D*). *E*, typical averaged and superimposed mIPSCs recorded in control conditions (thin line) and in the presence of 3 μM zolpidem (thick line). *F*, statistics of the zolpidem effect on the mean decay time constant (τ_{mean}). Zolpidem does not affect the value of slow decay time constant (τ_{slow}, *G*) but increases the percentage of this component (A_{slow}, *H*). Mean values were calculated from at least $n = 5$ cells. *Significant difference with respect to the control values.

10 μM concentration, this BDZ significantly prolonged the deactivation kinetics (relative $\tau_{mean} = 1.31 \pm 0.08$, $n = 8$, $P < 0.05$, Fig. 4*D*, *E*) and this effect was related to a prolongation of the slow deactivation component (relative $\tau_{slow} = 1.30 \pm 0.10$, $P < 0.05$; relative $\tau_{fast} = 1.18 \pm 0.10$, $P > 0.05$; and relative $A_{slow} = 0.81 \pm 0.24$, $n = 8$, $P > 0.05$). However, zolpidem (at concentrations up to 3 μM) had no significant effect on the deactivation kinetics of currents evoked by 10 mM GABA (Fig. 4*D* and *E*).

Notably, the deactivation kinetics observed following the application of 1 μM GABA ($\tau_{mean} = 328$ ms, Fig. 4*A* and *B*) had a much slower time course than that for short and saturating GABA pulses ($\tau_{mean} = 36.13$ ms, Fig. 4*B*) and also had a dramatically stronger sensitivity to flurazepam (compare Fig. 4*C* and *E*). Moreover, while

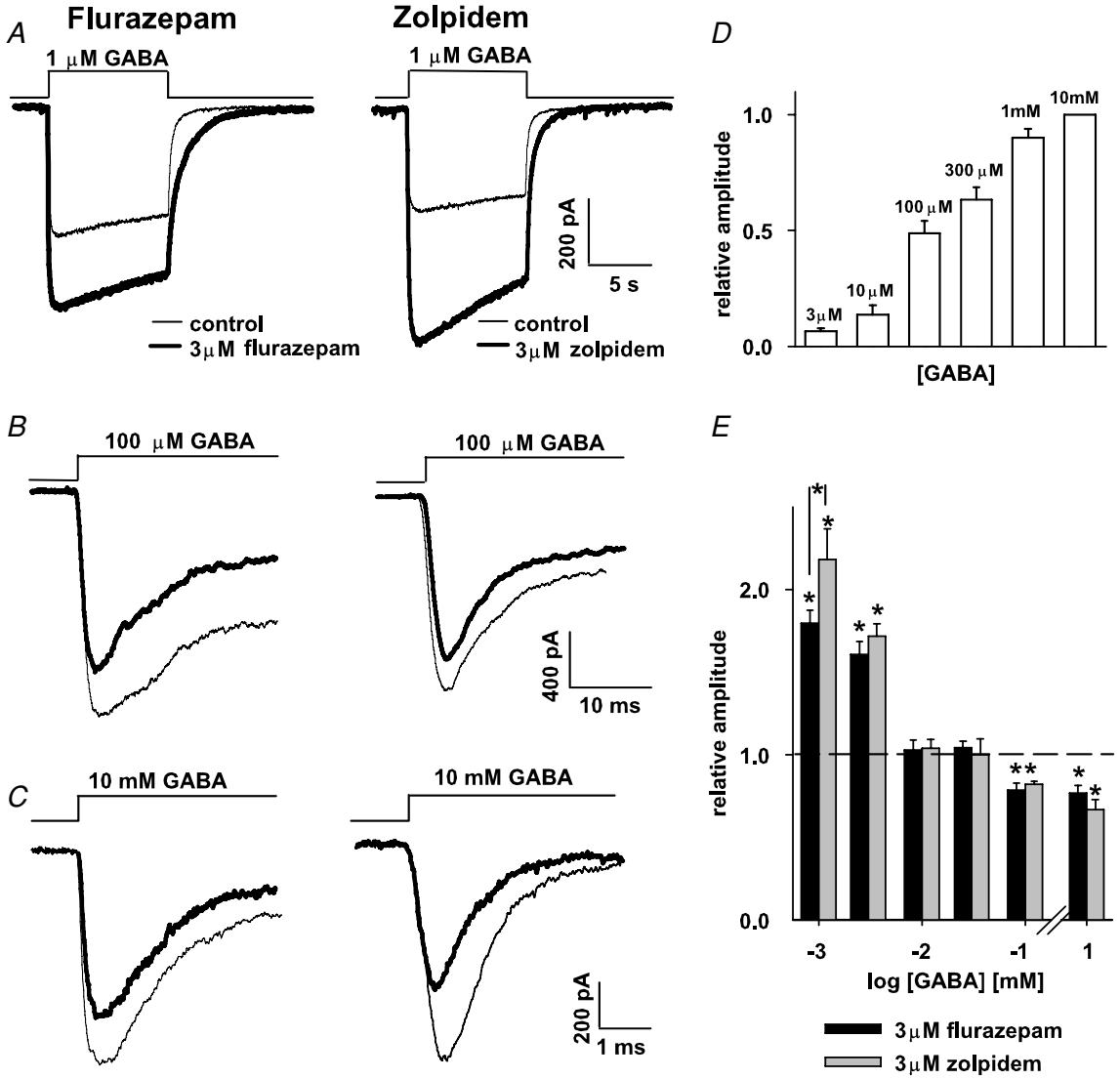

Figure 3. Effect of flurazepam and zolpidem on current amplitudes responses strongly depend on GABA concentration

Left column shows examples of current responses evoked in control conditions (thin lines) and in the presence of 3 μM flurazepam (thick line) while middle column shows analogous examples for studies on the zolpidem effect. *A*, examples of currents evoked by 1 μM GABA in control conditions (thin lines), in the presence of 3 μM flurazepam (thick line, left panel) and 3 μM zolpidem (thick line, right panel). *B* and *C*, examples of currents elicited by 100 μM and by 10 mM GABA, respectively, in control conditions (thin line) and in the presence of flurazepam (thick line, left) and zolpidem (thick line, right). *D*, normalized dose–response relationship for current amplitudes in control conditions. Duration of GABA application was sufficient for current to reach the peak. Mean values were calculated from at least $n = 4$ cells. *E*, statistics on zolpidem and flurazepam effects on the amplitudes of currents evoked by different GABA concentrations. Mean values were calculated from at least $n = 8$ cells. Insets above current traces depict the time course of applied agonist. *Significant difference with respect to the control values.

deactivation of currents elicited by 1–3 ms pulse of 10 mM GABA was not affected by zolpidem, deactivation following a long pulse of 1 μM GABA was strongly prolonged by this drug (compare Fig. 4*A* and *C* with *D* and *E*). These results indicate that both flurazepam and zolpidem might be involved in modulation of deactivation kinetics but their effects were critically dependent on conditions in which the receptors were activated. In particular, it seems important to elucidate how the effects of flurazepam and zolpidem depend on the time duration of the GABA pulse and on the extent of receptor desensitization.

Flurazepam and zolpidem affect the deactivation–desensitization coupling differently

It is known that prolonged exposure of $GABA_A$Rs to free agonist may result in a favoured entry into the desensitized state and therefore the time of agonist application may affect the deactivation kinetics (Jones & Westbrook, 1995). Taking into account this prediction, we have checked how time of agonist application affected deactivation kinetics of currents evoked by application at different [GABA] and how the ensuing deactivation–desensitization coupling is affected by flurazepam and zolpidem. To assess the deactivation–desensitization coupling at a given GABA concentration, currents were elicited by a GABA pulse of sufficient duration to reach the peak or a plateau value (a so called 'short' pulse; pulse durations are described in the legend of Fig. 5*A*–*C*) and then by a pulse at least five times longer ('long' pulses, Fig. 5*A*–*C*). The averaged deactivation time constants were measured for responses evoked by 'long' (τ_{mean}(long)) and 'short' pulses (τ_{mean}(short)) and the ratio of these time constants is shown in Fig. 5*D*. Interestingly, up to 10 μM GABA, the prolongation of agonist pulse did not affect the

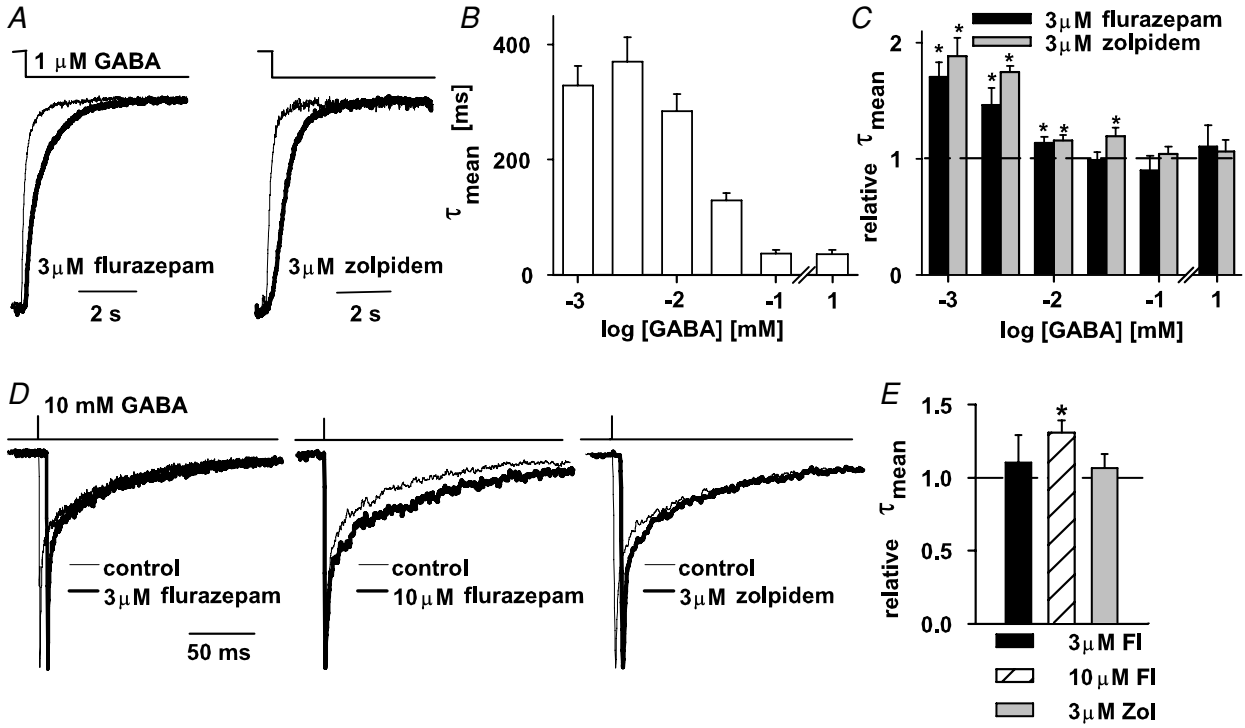

Figure 4. Flurazepam and zolpidem affect the deactivation kinetics
A, examples of normalized and superimposed traces evoked by 1 μM GABA (2 s application) in control conditions (thin lines) and in the presence of 3 μM flurazepam (thick line, left) or 3 μM zolpidem (thick line, right). *B*, dependence of mean deactivation time constant (τ_{mean}) on GABA concentration. Mean values were calculated from at least $n = 5$ cells. *C*, relative mean deactivation time constants in the presence of 3 μM flurazepam (black bars) and 3 μM zolpidem (grey bars) *versus* GABA concentration. Mean values were calculated from at least $n = 5$ cells. Note that a significant effect of both BDZ receptor agonists is observed only for GABA concentrations considerably below the EC_{50} value. *D*, examples of normalized responses elicited by short applications of saturating GABA (10 mM for 3 ms) in control conditions (thin line) and in the presence of flurazepam (thick lines, left and middle for 3 and 10 μM, respectively) and 3 μM zolpidem (thick line, right). Since at 3 μM flurazepam and 3 μM zolpidem there was no visible effect on the deactivation time course, the control traces were shifted to the left with respect to those recorded in the presence of flurazepam and zolpidem. *E*, statistics of the flurazepam and zolpidem effect on mean deactivation time constant (τ_{mean}). Insets above current traces depict the time course of the applied agonist. Mean values were calculated from at least $n = 6$ cells. *Significant difference with respect to the control values.

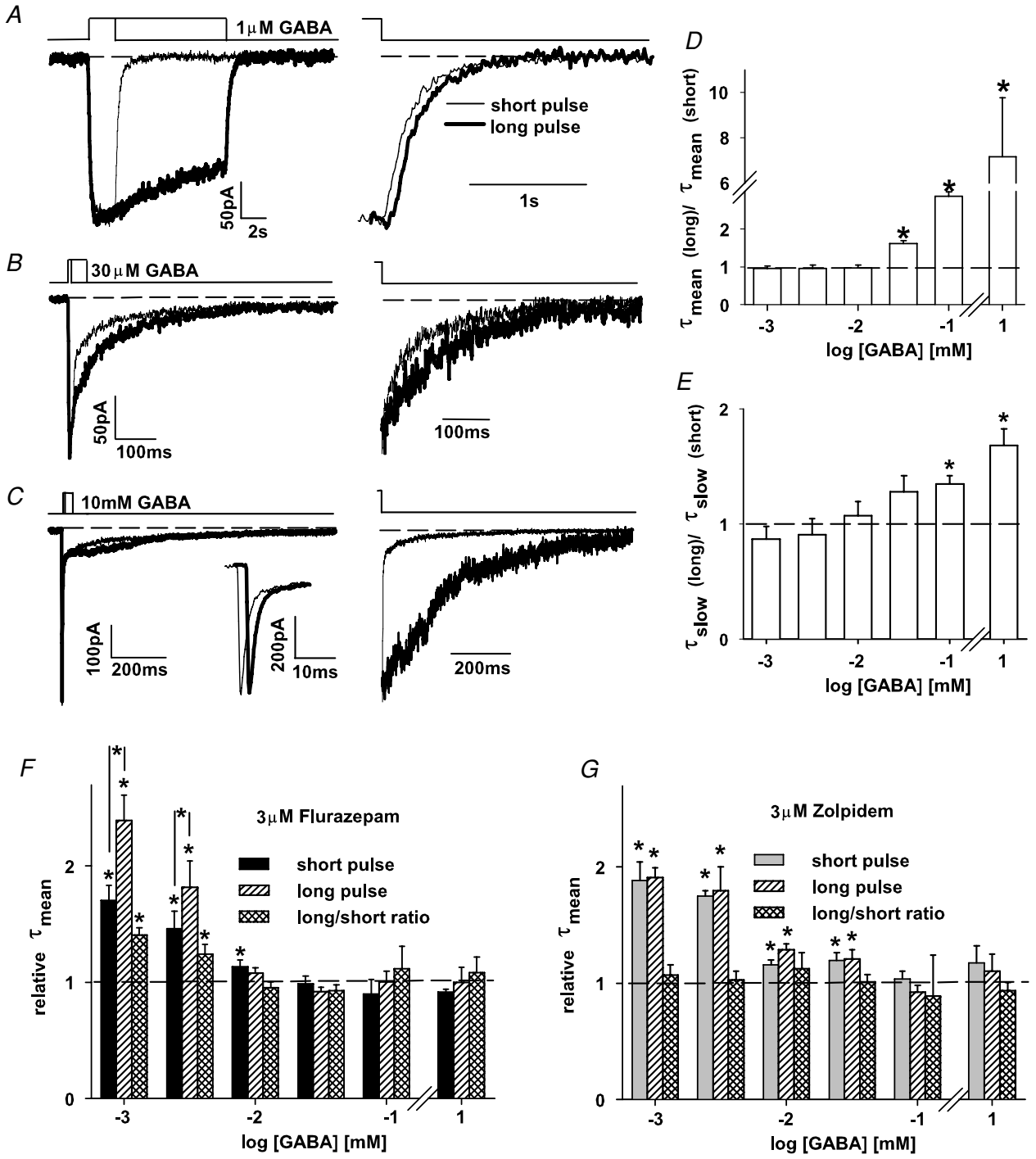

Figure 5. Flurazepam and zolpidem differentially affect the deactivation–desensitization coupling
A, *B* and *C*, examples of current traces evoked by 1 μM, 30 μM and 10 mM GABA, respectively, applied for short (thin line) and long time (thick line). The following durations of agonist applications were used at different [GABA]: 1 μM, 2 s; 3 μM, 1 s; 10 μM, 50 ms; 30 μM, 10 ms; 100 μM, 5 ms; 10 mM, 1 ms and the respective long applications were five times longer (see text for explanation of how short and long application times were defined). Right panels in *A*, *B* and *C* show the same current traces but normalized to the current value at the end of GABA pulse. In *C*, the inset shows the rapid phases of current responses on an expanded time scale. Note that at high [GABA] the deactivation following brief GABA pulse is much faster than that after a long application (mainly due to a predominant rapid deactivation component in the former one). *D*, the ratio of τ_{mean} values measured for deactivation currents following long and brief GABA applications, respectively, *versus* GABA concentration. Note that pulse duration affects τ_{mean} only for GABA concentrations equal or above 30 μM at which rapid desensitization

deactivation kinetics (Fig. 5*D*). On the contrary, starting from a GABA concentration of 30 μM, the larger [GABA] was, the larger the impact of time duration of the agonist pulse on the deactivation time course was (Fig. 5*D*). At high [GABA], a prolongation of the agonist pulse resulted in a reduction (or even disappearance) of the fast deactivation component (Fig. 5*C*) giving rise to a substantial increase in τ_{mean} (Fig. 5*D*). Indeed, while following 1 ms pulse of 10 mM GABA, there was a predominant fast component of ~2.6 ms (see section above and Fig. 5*C*); after a longer pulse (10–30 ms), such a fast component was absent (Fig. 5*C*, right panel). Interestingly, the rapid decay component in currents evoked by 1 ms (10 mM GABA) and during a long pulse of the same [GABA] had indistinguishable fast decay components (see inset on the expanded time scale in Fig. 5*C*) indicating that the apparent fast deactivation is due to a rapid desensitization process. Indeed, as presented in details in the next section, the value of the fast desensitization time constant was not significantly different ($P > 0.05$) from the fast deactivation component. The view that the fast deactivation component is due to rapid desensitization is further supported by the fact that the recovery in the paired-pulse experiments with a short gap (two 1 ms pulses of 10 mM GABA separated by a 5 ms gap) yielded a recovery at the limit of detection level (below 5%, data not shown). Moreover, as already mentioned, the time courses of current responses elicited by 10 mM GABA pulses of duration between 1 and 3 ms were indistinguishable (data not shown) which is consistent with the mechanism in which even a very short pulse (e.g. 1 ms) of saturating [GABA] is sufficient to induce a profound desensitization that determines the fast component of apparent deactivation. However, the prolongation of deactivation process (at high [GABA]) by increasing the pulse duration was not only due to reduction or disappearance of the rapid component. For half-saturating or saturating [GABA], the slow deactivation components were significantly longer than the ones following the short pulses (Fig. 5*E*). Thus, at high [GABA], rapid desensitization appears to play a dual role in shaping the deactivation time course: (i) it largely determines the fast component of apparent deactivation and (ii) sojourns into the desensitized states prolong the slow component of deactivation process (Jones & Westbrook. 1995).

We next checked how flurazepam and zolpidem affected the deactivation–desensitization coupling described above. As shown in Fig. 5*F*, flurazepam exerted a significantly stronger action on deactivation kinetics for currents evoked by long agonist pulses but this effect was present only at low [GABA] (1 and 3 μM). On the contrary, zolpidem did not affect the deactivation–desensitization coupling over the entire considered range of [GABA] (Fig. 5*G*).

Effect of flurazepam and zolpidem on kinetics of current responses elicited by saturating [GABA]

At saturating GABA concentrations, the binding step is expected to occur much faster than the conformational transitions between bound states. For this reason, the time course of current responses to saturating [GABA] is largely determined by gating properties of $GABA_AR$. Thus, in order to assess the impact of flurazepam and zolpidem on $GABA_AR$ gating, a series of application protocols for saturating [GABA] was employed. The effect of flurazepam and zolpidem on deactivation kinetics of currents evoked by short pulses (1–3 ms) of 10 mM GABA was presented in previous sections (Figs 4 and 5). As already mentioned, the deactivation time course of GABAergic currents has been found to strongly depend on the desensitization kinetics (Jones & Westbrook, 1995) and therefore it is important to examine the effects of these BDZ receptor agonists on the desensitization time course. To this end, current responses were evoked by prolonged (30–100 ms) applications of saturating [GABA] (Fig. 6*A*). In control conditions, the desensitization time constant (τ_{des}) was 2.05 ± 0.19 ms ($n = 26$) and the steady-state to peak (ss/peak) was 0.18 ± 0.023 ($n = 26$). Flurazepam at concentrations of 1–10 μM had no significant effect either on τ_{des} or on the steady-state to peak ratio (Fig. 6*B* and *C*). In contrast, zolpidem was found to significantly increase the rate but not the extent of desensitization (at 3 μM zolpidem relative τ_{des}: 0.83 ± 0.04, $P < 0.05$, and relative ss/peak: 0.89 ± 0.07, $n = 10$, $P > 0.05$, Fig. 6*B* and *C*). This result appears surprising as flurazepam (at 10 μM) prolonged current deactivation while zolpidem did not.

It is known that $GABA_AR$s tend to strongly accumulate in the desensitized state even after a brief exposure

becomes prominent. *E*, ratio of time constants of slow deactivation components (τ_{slow}) measured following long and brief GABA applications, respectively, *versus* [GABA]. In *D* and *E*, mean values were calculated from at least $n = 8$ cells. *F*, relative τ_{mean} for deactivation currents in the presence of 3 μM of flurazepam (with respect to control conditions) for short pulses (black bars) and for long pulses (middle hatched bars). Cross-hatched bars show the ratios of relative τ_{mean} values measured for long and short pulses, respectively. *G*, analogous results as presented in *F* but obtained for 3 μM of zolpidem. In *F* and *G*, mean values were calculated from at least $n = 5$ cells. Note that at low [GABA] flurazepam enhances the deactivation–desensitization coupling and zolpidem does not. Insets above current traces depict the time course of applied agonist. *Significant difference with respect to the control values.

to high [GABA] (Jones & Westbrook, 1995; see also section on deactivation–desensitization coupling). This phenomenon can be visualized in the paired-pulse experiments (Fig. 6*D* and *E*). The fact that the amplitude of response to the second pulse is smaller than that for the first GABA application, indicates that a considerable proportion of $GABA_A$Rs accumulated in the desensitized state. As shown in Fig. 6*D* and *E*, both flurazepam and zolpidem significantly reduced the recovery parameter *R* (see Methods) but the effect of zolpidem was clearly stronger ($P < 0.05$). The reduction of recovery by these BDZ receptor agonists might be due to an increase in desensitization rate and/or the reduction of the resensitization rate constants but it cannot be excluded

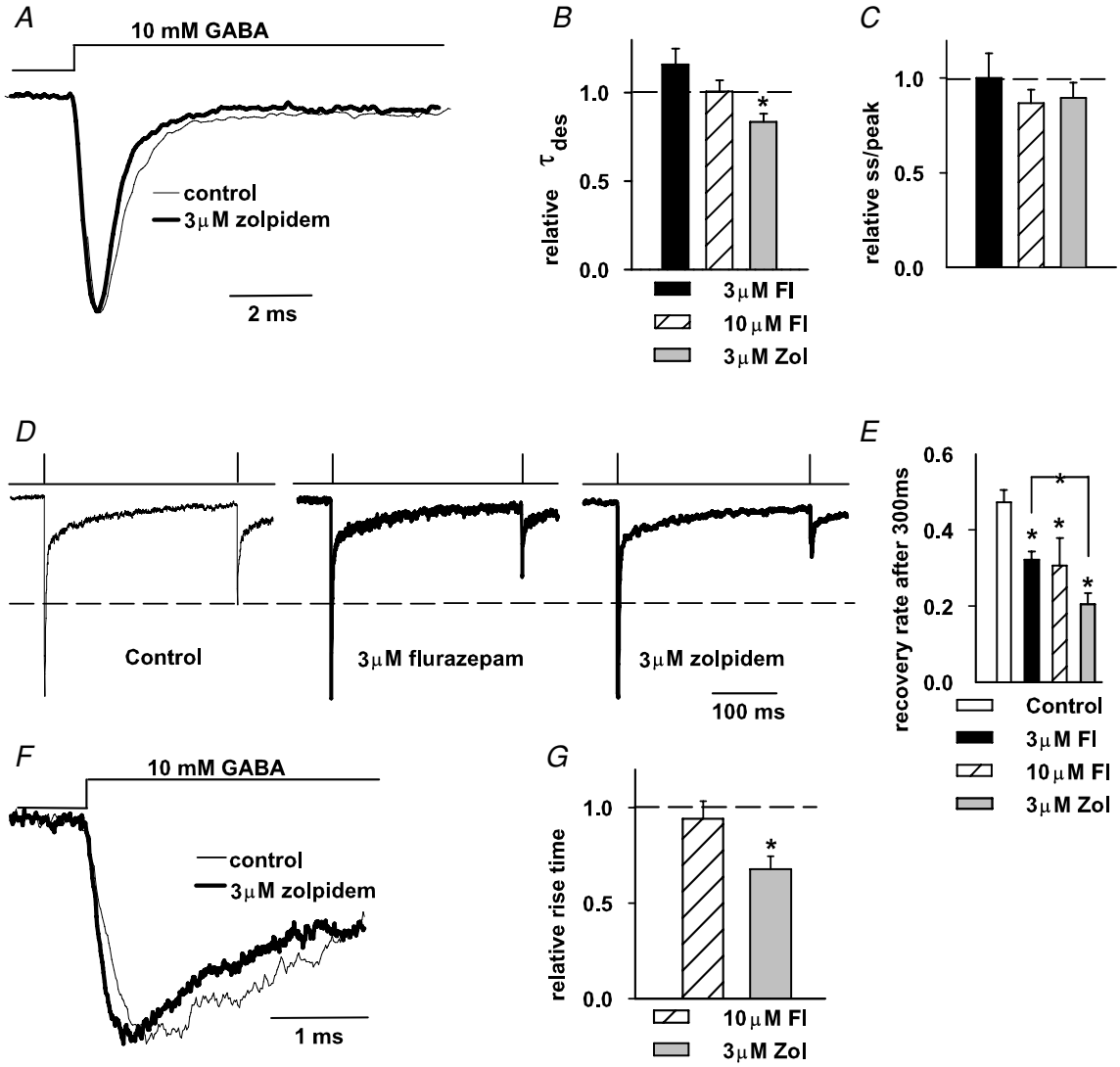

Figure 6. Flurazepam and zolpidem affect the time course of responses elicited by saturating [GABA]
A, normalized and superimposed current traces evoked by prolonged application of saturating (10 mM) [GABA] in control conditions (thin line) and in the presence of 3 μM zolpidem (thick line). *B* and *C*, statistics of flurazepam and zolpidem effects on the time constant of desensitization onset (τ_{des}, *B*) and on the steady-state to peak parameter (ss/peak, *C*). In *B* and *C*, mean values were calculated from at least $n = 6$ cells. *D*, examples of normalized currents recorded in the paired-pulse experiments (3 ms applications of 10 mM GABA separated by 300 ms time interval) in control conditions (thin line, left) and in the presence of 3 μM flurazepam (thick like, middle) and 3 μM zolpidem (thick line, right). Dashed line indicates the amplitude of response to the second pulse in control conditions. *E*, statistics of flurazepam and zolpidem effects on the recovery parameter *R* (see Methods). Mean values were calculated from at least $n = 6$ cells. *F*, normalized and superimposed current traces evoked by saturating GABA in control conditions (thin line) and in the presence of 3 μM zolpidem (thick line). *G*, statistics of the flurazepam and zolpidem effects on 10–90% rise time. Mean values were calculated from at least $n = 4$ cells. Insets above current traces depict the time course of applied agonist. *Significant difference with respect to the control values.

that other parameters (such as, for example, unbinding rate) could also be involved.

In control conditions, the 10–90% rise time of current responses evoked by rapid application of 10 mM GABA was 0.23 ± 0.01 ms ($n = 16$). Flurazepam at a concentration up to 10 μM did not significantly affect the current onset rate (Fig. 6*G*). In contrast, zolpidem at a concentration of 3 μM significantly accelerated the 10–90% rise time (relative 10–90% rise time 0.66 ± 0.06, $n = 6$, $P < 0.05$, Fig. 6*F* and *G*).

Altogether, recordings of currents elicited by various protocols of saturating [GABA] applications revealed that both flurazepam and zolpidem affected the time course of recorded responses although their effects showed qualitative differences.

Effect of BDZ receptor agonists on current responses to brief applications of half-saturating [GABA]

The results presented above show that both mIPSCs and current responses elicited by exogenous GABA applications are potently modulated by BDZ receptor agonists but a correlation between these effects is not straightforward. While these compounds markedly decreased the amplitude of current responses to 100 μM and 10 mM GABA (Fig. 3), the same concentrations of these drugs clearly increased the mIPSC amplitudes (Fig. 1). This observation is particularly puzzling because the peak of synaptic GABA transient is commonly believed to reach millimolar concentrations (Clements, 1996; Mozrzymas *et al.* 1999, 2003*b*; Overstreet *et al.* 2002; Mozrzymas, 2004). Moreover, while both flurazepam and zolpidem prolonged mIPSC decaying phase (Fig. 2), a moderate effect on deactivation of current responses was observed only for 10 μM flurazepam (Fig. 4). There are at least two possible explanations for these differences: (i) activation of synaptic receptors is evoked by a GABA transient that is considerably shorter than GABA pulses applied using our application system, and (ii) synaptic $GABA_A$Rs are endowed with qualitatively different properties with respect to the extrasynaptic ones that are likely to be abundantly present in the excised patches (or whole cells). In the present section, we shall consider the first possibility while the second one is discussed in the next one. As mentioned in Methods, the shortest pulse duration that can be reliably applied with our perfusion system was ~0.8–1 ms. Thus, the major problem with experimental modelling of synaptic transient is that it may be up to one order of magnitude shorter than 1 ms (Mozrzymas *et al.* 1999, 2003*b*; Overstreet *et al.* 2002; Mozrzymas, 2004). Most importantly, such extremely short duration of the agonist pulse makes a relatively high (millimolar) synaptic GABA concentration considerably distant from saturation. In practice, the amplitude of the postsynaptic current response to a synaptic agonist pulse is better correlated with the product of the peak concentration and time duration of neurotransmitter presence in the cleft rather than with the peak agonist concentration alone (see, for example, Mozrzymas, 2004; Barberis *et al.* 2004). Thus, as an attempt to qualitatively reproduce the synaptic conditions, we have applied the agonist for 1 ms while the concentration of GABA was set at 100 μM. In this way, the product of peak concentration and application time is similar to that estimated for synaptic transient (Overstreet *et al.* 2002; Mozrzymas *et al.* 2003*b*; Mozrzymas, 2004) while peak agonist concentration and time exposure were proportionally rescaled. It is expected that current responses to 1 ms application of 100 μM GABA would result in the activation of only a portion of receptors available in the patch because during such a short exposure to non-saturating [GABA], only a fraction of receptors would complete the binding step. To test this prediction, the amplitude of response to 1 ms application of 100 μM GABA was compared with that measured for 5 ms exposure to the same GABA concentration (Fig. 7*A* and *B*). The application of 100 μM GABA for 5 ms is sufficient for current to reach its maximum value (Fig. 7*A*, see also Mozrzymas *et al.* 2003*a*). On average, in control conditions, the ratio of peaks evoked by 1 and 5 ms was 0.31 ± 0.04 ($n = 6$, Fig. 7*A* and *B*) indicating that in this case the key mechanism of non-saturation is to 'trim' the receptor activation by agonist removal after a short exposure. As shown in Fig. 7*A* and *B*, the peak ratios (measured for 1 and 5 ms GABA applications) were clearly increased by both flurazepam and zolpidem (0.64 ± 0.07, $n = 6$ and 0.60 ± 0.04, $n = 7$, for 3 μM flurazepam and 3 μM zolpidem, respectively, $P < 0.05$). Interestingly, while both BDZ receptor agonists decreased the amplitudes of currents evoked by 5 ms application of 100 μM GABA (Fig. 7*A* and *B*, see also Fig. 3*C* and *D*), the amplitudes of currents evoked by 1 ms application of the same GABA concentrations were enhanced by flurazepam and zolpidem (Fig. 7*A*, see dashed line). This result shows that in conditions similar to those that presumably take place in the synapse, BDZ receptor agonists may potentiate the responses elicited by the same GABA concentration for which currents evoked by longer applications are down-regulated by these drugs (compare Figs 3 and 7).

The observed increase in peak ratios for responses to 1 and 5 ms of 100 μM GABA in the presence of flurazepam and zolpidem (Fig. 7) appears compatible with the increase in the binding rate by these drugs. Effective rate of agonist binding is assumed to be proportional to the agonist concentration ($\sim k_{on}$[GABA], where k_{on} is the binding rate) and at a non-saturating [GABA] (100 μM GABA is far from saturating the onset rate, Mozrzymas *et al.* 2003*a*; Fig. 3*D*) the current onset is expected to depend on the binding rate. We therefore analysed the onset kinetics for responses evoked by 5 ms application of 100 μM GABA and found that in control conditions, the 10–90% rise time

was 1.82 ± 0.06 ms ($n = 9$). The onset kinetics of these currents was significantly accelerated both by flurazepam and zolpidem (relative 10–90% rise time 0.88 ± 0.07, $n = 5$, and 0.85 ± 0.03, $n = 4$, for 3 μM flurazepam and 3 μM zolpidem, respectively, $P < 0.05$, Fig. 7*C* and *D*).

Responses of recombinant ('synaptic') $\alpha 1\beta 2\gamma 2$ receptors to brief applications of non-saturating [GABA] show similar zolpidem sensitivity as mIPSCs

As already mentioned, mIPSCs and current responses to brief GABA applications may differ in their BDZ sensitivity because excised patches might contain a considerable proportion of extrasynaptic receptors characterized by different kinetics and pharmacology with respect to synaptic channels (e.g. Farrant & Nusser, 2005). A functional indication for such a difference is the fact that the time course of synaptic currents clearly differs from that observed for current responses to brief agonist applications (e.g. Jones & Westbrook, 1995; Mozrzymas *et al.* 1999, 2003*b*). In order to test whether different BDZ action on mIPSC and on current responses might result from differences between synaptic and extrasynaptic receptors, we have repeated key protocols for currents

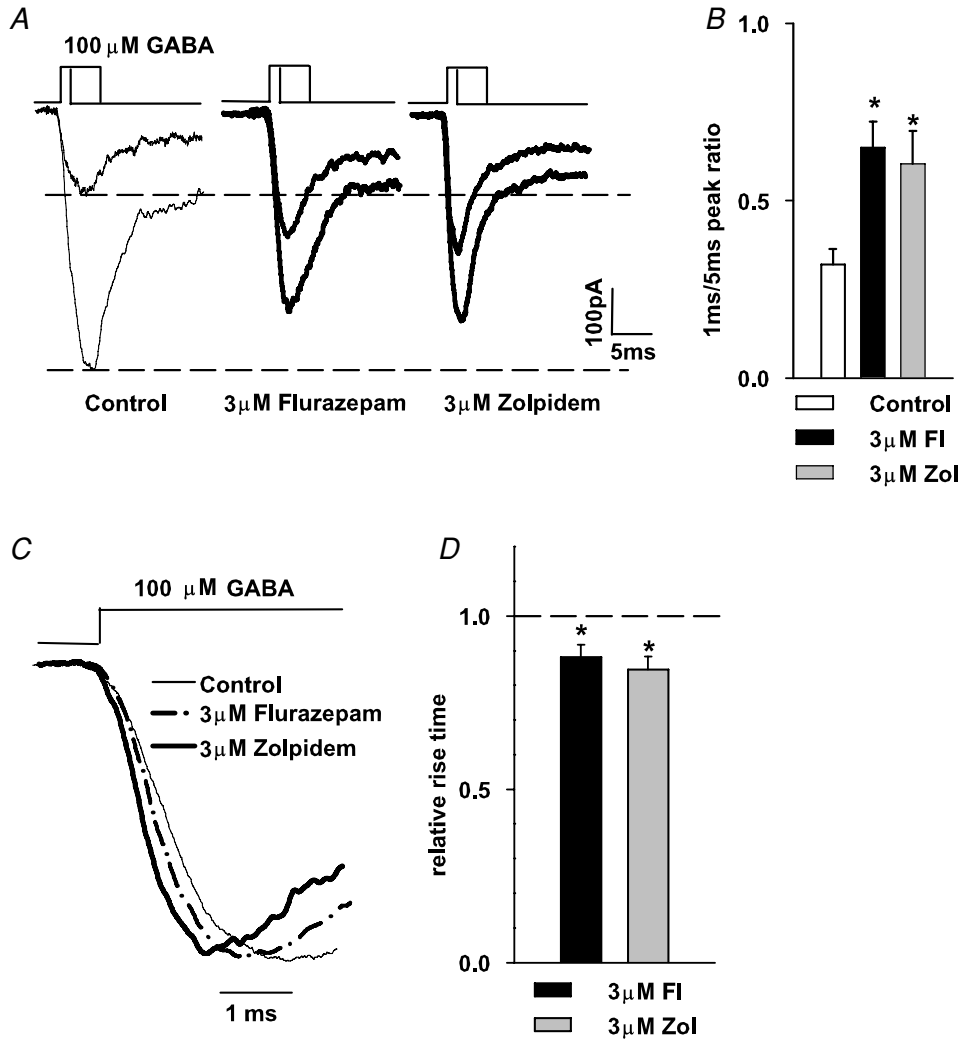

Figure 7. Timing of responses to non-saturating [GABA] determines the impact of BDZ receptor agonist on current amplitudes
A, examples of superimposed currents evoked by 100 μM GABA applied for 1 and 5 ms in control conditions (thin lines, left) and in the presence of 3 μM flurazepam (thick line, middle) and 3 μM zolpidem (thick line, right). *B*, statistics of flurazepam and zolpidem effects on amplitude ratio (amplitude of current evoked by 1 ms pulse/amplitude of current evoked by 5 ms pulse). Mean values were calculated from at least $n = 6$ cells. *C*, examples of normalized current responses evoked by 100 μM GABA in control conditions (thin line) and in the presence of 3 μM flurazepam (thick dash–dotted line) or 3 μM zolpidem (thick line). *D*, statistics of flurazepam and zolpidem effects on the 10–90% rise time of currents evoked by 100 μM GABA. Mean values were calculated from at least $n = 4$ cells. Insets above current traces depict the time course of applied agonist. *Significant difference with respect to the control values.

mediated by recombinant $\alpha1\beta2\gamma2$ receptors. This receptor subtype is abundantly expressed in the CNS (Whiting, 2003) and, most importantly, in several brain regions is predominant in GABAergic synapses. As already reported in our previous study (Mercik *et al.* 2007), both flurazepam and zolpidem reduced the amplitudes of current responses to saturating [GABA] by 15 and 31%, respectively, which is close to what we report now for neurons (Fig. 3). In the context of the BDZ effect on synaptic currents, it is particularly interesting to repeat the protocol depicted in Fig. 7 (brief and long application of 100 μM GABA). As shown in Fig. 8*A* and *B*, the presence of 3 μM zolpidem significantly increased the peak ratio, similarly to the case of responses mediated by neuronal receptors (Fig. 7). The only difference between responses mediated by neuronal and recombinant receptors was that the activation of the latter by application of 100 μM GABA, yielded currents characterized by a faster onset rate (10–90% rise time for $\alpha1\beta2\gamma2$-mediated currents was 1.33 ± 0.13 ms, $n = 4$) and for this reason the short application in the protocol in Fig. 8*A* was reduced to 0.8 ms.

It is known that the kinetic and pharmacological properties of synaptic (e.g. $\alpha1\beta2\gamma2$) and of extrasynaptic $GABA_A$Rs can be substantially different. While synaptic $GABA_A$Rs respond to rapid (phasic) pulses of agonist, extrasynaptic ones experience prolonged exposures to ambient [GABA] in a submicromollar range (e.g. Farrant & Nusser, 2005). It is thus interesting to confront the strong BDZ sensitivity of deactivation process in current responses to low [GABA] recorded from neurons to that measured from HEK cells expressing $\alpha1\beta2\gamma2$ receptors. To this end we have investigated the zolpidem ($\alpha1$-preferring BDZ receptor agonist) effect on current responses mediated by $\alpha1\beta2\gamma2$ receptors and elicited by 1 μM GABA. As shown in Fig. 8*C* and *D*, zolpidem strongly enhanced the current amplitude (relative amplitude

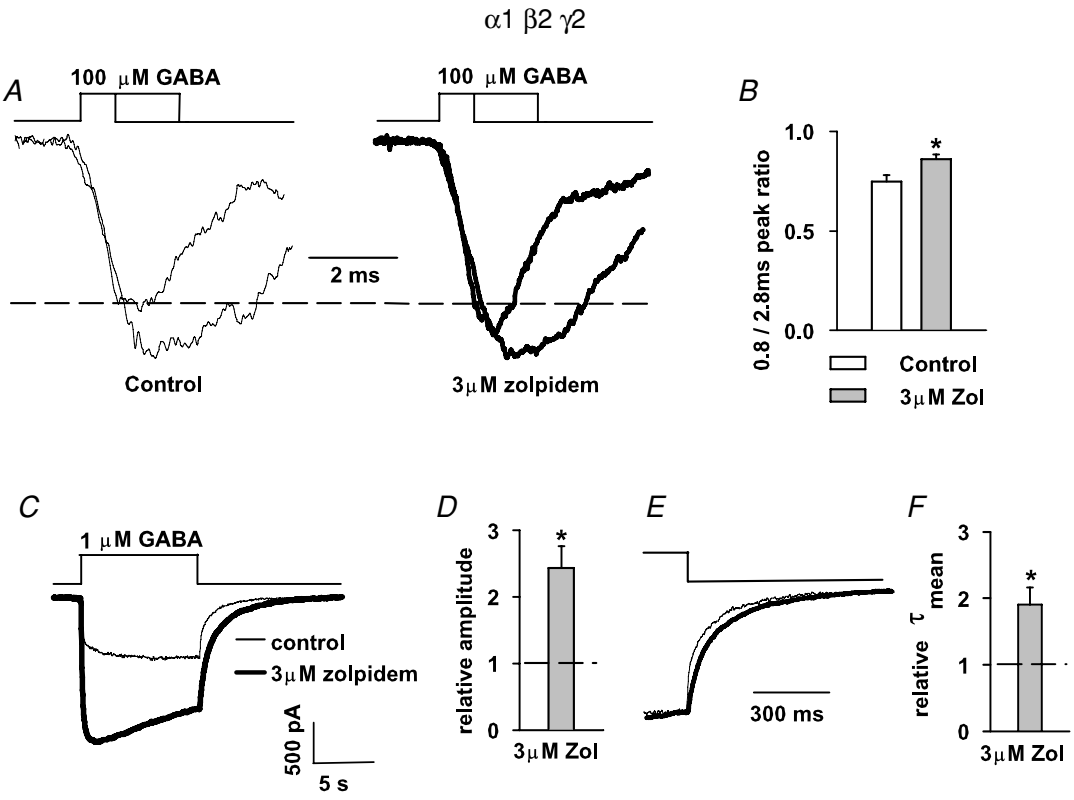

Figure 8. Effects of BDZ receptor agonists on neuronal $GABA_A$ receptors are reproduced for recombinant $\alpha1\beta2\gamma2$ receptors

A, examples of normalized and superimposed currents evoked by 100 μM GABA applied for 0.8 and 2.8 ms in control conditions (thin lines, left) and in the presence of 3 μM zolpidem (thick line, right). *B*, statistics of the zolpidem effect on amplitude ratio (amplitude of current evoked by 0.8 ms pulse/amplitude of current evoked by 2.8 ms pulse). Mean values were calculated from at least $n = 6$ cells. *C*, examples of currents evoked by 1 μM GABA in control conditions (thin line) and in the presence of 3 μM zolpidem (thick line). *D*, statistics of zolpidem effect on amplitude of currents evoked by 1 μM GABA. Mean value was calculated from $n = 7$ cells. *E*, normalized and superimposed traces presented in *C*. A prolongation of deactivation by zolpidem (thick line) is clearly seen. *F*, statistics of zolpidem effects on the mean deactivation time constant (τ_{mean}). Mean value was calculated from $n = 4$ cells. Insets above current traces depict the time course of an applied agonist. *Significant difference with respect to the control values.

2.42 ± 0.33, $n = 7$) and this effect was similar to that observed in neurons (Fig. 4*A* and *C*). Moreover, the mean deactivation time constant (τ_{mean}) was clearly prolonged by zolpidem (relative $\tau_{mean} = 1.90 \pm 0.25$, $n = 4$, Fig. 8*E* and *F*), nicely reproducing our observations in neurons (Fig. 4*A* and *C*). These experiments indicate that in the considered model of cultured hippocampal neurons, the BDZ sensitivity of currents elicited by exogenous GABA, qualitatively follows the pattern observed for responses mediated by recombinant $\alpha 1\beta 2\gamma 2$ receptors.

Model simulations

The effects of flurazepam and zolpidem on mIPSCs and on current responses to exogenous GABA applications suggest that these BDZ receptor agonists modulate both binding and gating of $GABA_AR$. A robust enhancement of responses to 1–3 μM GABA (Fig. 3) confirms that BDZs strongly up regulate the agonist binding. It might be suggested that a similar effect could also be reproduced by increasing the receptor efficacy (Rusch & Forman, 2005; Downing *et al.* 2005; Campo-Soria *et al.* 2006). However, it would lead to an increase in amplitude at saturating [GABA], contrary to our experimental findings (Fig. 3, see also Discussion).

Both flurazepam and zolpidem clearly modulated the time course of current responses elicited by saturating [GABA] indicating that they affected gating properties of neuronal $GABA_AR$s. The reduction of amplitudes of responses evoked by saturating and half-saturating [GABA] (Fig. 3) suggests an increased occupancy of desensitized state(s). Enhancement of the desensitization time constant by zolpidem (Fig. 6*A* and *B*) together with a slower recovery observed for both compounds (Fig. 6*D* and *E*) further suggest this possibility. It is worth noting that strong reduction of the recovery by BDZ receptor agonists (Fig. 6*E*) could result in altered frequency dependence of IPSCs (Mellor & Randall, 1997). In our previous report (Mozrzymas *et al.* 2003*b*) we have proposed that modulation of desensitization may offer a potent mechanism of an amplitude regulation of responses evoked by saturating [GABA]. Clearly, interpretation of our macroscopic data based on such a mechanism requires a widely accepted notion that single channel conductance is not affected by these compounds (Twyman *et al.* 1989; Vicini *et al.* 1987; Rogers *et al.* 1994; Perrais & Ropert, 1999; but see Eghbali *et al.* 1997). A qualitatively similar enhancement of desensitization to that induced by zolpidem (Fig. 6*A* and *B*) was previously reported for other BDZs also by Lavoie & Twyman (1996) and Mellor & Randall (1997). The recovery process was slowed down by both flurazepam and zolpidem (Fig. 6*D* and *E*) and this effect could potentially involve enhanced desensitization rate, slower resensitization and/or a decrease in the unbinding rate. The enhancement of the onset rate for currents elicited by saturating [GABA] in the presence of zolpidem (Fig. 6) could suggest an increase in transition rate from closed to open bound states. However, as mentioned above, such an effect would result in enhancement of responses to saturating [GABA], contrary to our data. An alternative possibility could be that the acceleration of current onset was due to increased desensitization rate (Mozrzymas *et al.* 2003*a*). We suggest that in the case of zolpidem, a concomitant observation of accelerated desensitization onset, a decrease in current amplitude for responses to saturating [GABA], acceleration of current onset and reduced recovery in the paired-pulse experiments (Fig. 6) indicate that this BDZ receptor agonist does affect the desensitization kinetics. Although the impact of flurazepam was weaker, it is tempting to assume that the major effects of both drugs (a decrease in amplitude of current response to saturating [GABA] and slow down in recovery) resulted from increased occupancy in desensitized conformation(s). However, the present data are not sufficient to precisely indicate, for example, which of potentially several desensitized states are affected by the considered BDZ receptor agonists. Moreover, it needs to be born in mind that alteration of any current characteristics may potentially result from a change in any rate constant in the considered gating scheme because all receptor conformations are functionally coupled (Colquhoun, 1998; Mozrzymas *et al.* 2003*a*). Nevertheless, we made an attempt to use model simulations to interpret, at a qualitative level, our major findings. The main goal was to consider the impact of specific non-equilibrium conditions of synaptic receptor activation on the susceptibility of mIPSCs to modulation by BDZ agonists. Moreover, using this approach we tried to evaluate to what extent differences in BDZ modulation of mIPSCs and of current responses result from different conditions in which these currents are activated.

Taking into account the available experimental evidence, our strategy was to propose a minimum requirement for BDZ effects by adapting a previously published model. For this purpose we have used the model proposed in Mozrzymas *et al.* (2003*b*) (Fig. 9*A*) in which we have slightly increased the binding rate in control conditions because in the present study the currents elicited by 100 μM yielded currents with faster onset. The major simplifying assumption of this model is that it postulates only one set of fully bound states (open, closed, desensitized). In particular, it is known that besides the fast desensitized state included in the model, there could be several slower desensitized components which might additionally shape the current responses, especially those elicited by long GABA pulses. The major effect of BDZ was to increase (by nearly 80%, Fig. 3) the currents evoked by 1 μM GABA. Such a BDZ effect can be easily reproduced by increasing the binding rate

k_{on} from 8.0 to 12.0 ms^{-1}. At saturating (10 mM) and at 100 μM [GABA], both BDZ receptor agonists considered here reduced the current amplitude (Fig. 3) indicating that, as mentioned above, both flurazepam and zolpidem favour the occupancy of the desensitized state. As an attempt to model this possibility, we assumed that BDZ increases the rate constant of entrance into the doubly bound desensitized state (d_2 from 12 to 17 ms^{-1}). The fact that BDZs do not affect opening/closing rates (Twyman *et al.* 1989; Rogers *et al.* 1994) provides additional, although indirect, evidence that the observed BDZs effects are due to their action on the desensitized state. It might be argued that a substantial modification of desensitization kinetics could affect the burst durations, the effect that has not been observed by Twyman *et al.* (1989) and Rogers *et al.* (1994). However, the impact of desensitization on the burst durations is expected to be best manifested at high agonist concentrations while single channel analysis in these studies was performed for low [GABA]. Thus, the effect of BDZs on burst durations at high [GABA] remains to be elucidated. The fact that the model considered in the present report is oversimplified leads to some predictions that were not supported by the experiment (e.g. an increase in d_2 would lead to prolongation of deactivation kinetics that was not consistently confirmed in the experiment). The reason for this discrepancy is not clear. It may be speculated that real $GABA_A$Rs are endowed with several desensitized conformations characterized by distinct kinetics and BDZs could affect a desensitized conformation with a slower kinetics, whose impact on deactivation would be minor.

Synaptic currents were simulated as responses to the exponentially decaying agonist waveform

$$(y(t) = A \exp(-t/\tau_{transient}),$$

where $A = 1.0$ mM and $\tau_{transient} = 0.1$ ms). As shown in Fig. 9*B*, modelling of BDZ action by increasing k_{on} and d_2 was sufficient to reproduce the increase in mIPSC amplitude. Using the same model, we have simulated the responses to 1 and 5 ms of 100 μM GABA. As shown in Fig. 9*C*, the above-mentioned assumptions were sufficient to qualitatively reproduce our finding that the peak ratio for currents evoked by 1 and 5 ms is clearly increased by BDZ (Fig. 7*A* and *B*). Moreover, while the absolute value of the response to 1 ms is increased by BDZ, the response to 5 ms is down regulated by this modulator (Fig. 9*C* and *D*). Thus, this simulation further indicates that there is no qualitative contradiction between observed enhancement of mIPSC by BDZ (Fig. 1) and a down-regulation of response to saturating or half-saturating [GABA] by this drug (Fig. 3).

Discussion

The major finding of the present work is that the non-equilibrium conditions of synaptic $GABA_A$R

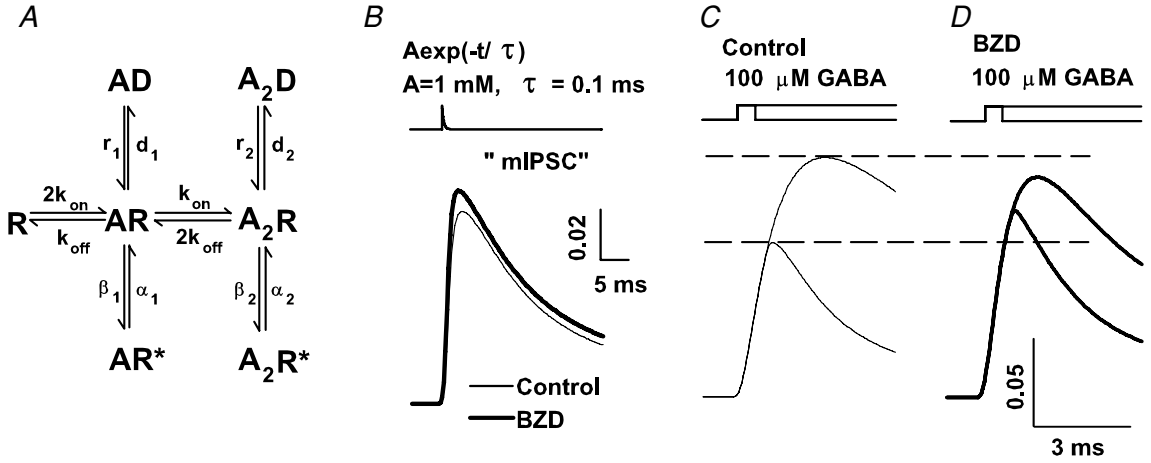

Figure 9. Increase in binding and desensitization rates qualitatively reproduces BDZ effects in model simulations

A, the frame of the model in Jones & Westbrook (1995). The rate constants were adapted from Mozrzymas *et al.* (2003*b*) with increased k_{on} value as explained in text. Simulations of currents in control conditions were made with the following rate constants: $k_{on} = 8.0$ ms^{-1} mM^{-1}, $k_{off} = 1$ ms^{-1}, $d_2 = 12$ ms^{-1}, $r_2 = 0.07$ ms^{-1}, $\beta_2 = 3.0$ ms^{-1}, $\alpha_2 = 0.4$ ms^{-1}, $d_1 = 0.045$ ms^{-1}, $r_1 = 0.014$ ms^{-1}; $\beta_1 = 0.15$ ms^{-1}, $\alpha_1 = 1.5$ ms^{-1}. The effect of BDZ was modelled by increasing k_{on} to 12.0 ms^{-1} mM^{-1} and d_2 to 17 ms^{-1}. Currents are simulated as a sum of open states occupancies. *B*, simulated synaptic currents in control conditions (thin line) and in the presence of BDZ (thick line). Synaptic GABA transient was modelled as exponentially decaying function: $A \exp(-t/\tau)$ where $A = 1.0$ mM and $\tau = 0.1$ ms. *C* and *D* show simulations of currents elicited by short (1 ms) and longer (5 ms) applications of 100 μM GABA in control conditions (thin lines, left) and in the presence of BDZ (thick lines, right). Note that response to short application is enhanced by BDZ while that elicited by longer GABA pulses was reduced. Insets above simulated traces described considered GABA application protocol.

activation, dictated by a very brief synaptic agonist transient (Clements, 1996; Mozrzymas *et al.* 1999, 2003*b*; Overstreet *et al.* 2002; Mozrzymas, 2004), have a crucial impact on IPSC sensitivity to BDZ receptor agonists. It was puzzling that mIPSCs were enhanced by flurazepam and zolpidem while responses evoked by saturating or half-saturating [GABA] were down-regulated by these drugs (Figs 1 and 3). This discrepancy could not result from different $GABA_AR$ subtypes in synapses and in excised patches (or whole cells) because a similar BDZ effect was observed for $\alpha1\beta2\gamma2$ receptors (Fig. 8; Mercik *et al.* 2007) that are abundantly present in GABAergic synapses (e.g. Farrant & Nusser, 2005). Moreover, our experimental data (Figs 7 and 8) and model simulations (Fig. 9) show that for non-saturating [GABA], appropriate timing of GABA applications is sufficient to reproduce such apparently opposite BDZ effects. This reflects a general rule that a very brief agonist transient renders the IPSCs particularly sensitive to modulators affecting GABA binding and the larger the distance from saturation, the larger the impact of such modifiers (Mozrzymas *et al.* 1999, 2003*b*; Mozrzymas, 2004).

As explained above, the most parsimonious mechanism for observed effects of flurazepam and zolpidem is an up-regulation of binding and desensitization. Although modulation of $GABA_AR$ gating by both BDZ receptor agonists considered here appears most compatible with enhancement of desensitization, there were qualitative differences in modulation of gating by flurazepam and zolpidem. The impact of these drugs on deactivation–desensitization coupling (Fig. 5) and on desensitization (Fig. 6) was clearly different. Our data are insufficient to ascribe these differences to, for example, differential modulation of distinct desensitized conformations. Moreover, we cannot exclude that these compounds additionally affected conformational transitions other than desensitization (e.g. open/closed) but for reasons presented in 'Model simulations' we believe that their impact is minor. The mechanism proposed here differs from that recently suggested by Rusch & Forman (2005) and Downing *et al.* (2005), who postulated enhancement in efficacy by BDZs in a spontaneously active $GABA_AR$ mutant. However, mutations rendering the receptors spontaneously active could affect also their sensitivity to BDZs. More recently, however, Campo-Soria *et al.* (2006) have observed that in oocytes expressing ultrahigh levels of wild type $GABA_ARs$, diazepam induced a detectable current that was considered as further evidence for the enhancement of $GABA_AR$ efficacy by diazepam. Although our data are not sufficient to discriminate between these mechanisms, there are some points that are worth pointing out. A substantial enhancement of receptor efficacy by BDZs would be expected to result in a modification of open and/or closed time distributions that has not been observed in classical single channel studies (Twyman *et al.* 1989; Rogers *et al.* 1994). Since the peak of response depends on the occupancy balance of closed, open and desensitized states, it is expected that the BDZs-induced increase in efficacy would increase the amplitude of current evoked by saturating [GABA]. However, our experiments show the opposite (Fig. 3). While there is a general agreement that modulation of receptor desensitization may critically shape the time course of GABAergic currents, the impact of this conformation has not been considered in the papers suggesting BDZ-induced enhancement of efficacy. Finally, we have made an attempt to record currents evoked by up to 10 μM flurazepam or to 3 μM zolpidem but no detectable currents were observed (in some cultures $GABA_AR$ expression was quite high: whole-cell responses to 1 μM GABA were above 1 nA at −50 mV with current resolution of ∼5–10 pA). It is thus possible that diazepam might act differently than flurazepam or zolpidem. Taking altogether, in our view, the works discussed above (Rusch & Forman, 2005; Downing *et al.* 2005; Campo-Soria *et al.* 2006) raise an interesting possibility that BDZs might affect the receptor efficacy but do not exclude their effect on binding or desensitization and the precise contributions of these mechanisms remain to be assessed.

It is surprising that both flurazepam and zolpidem strongly slowed down the deactivation of currents evoked by low (1–30 μM) GABA (Fig. 4) while for responses to higher [GABA] this effect was not present (except for a subtle effect at 10 μM flurazepam). Importantly, this pattern was observed both for neuronal $GABA_ARs$ and recombinant $\alpha1\beta2\gamma2$ receptors (Figs 3 and 8). It is still more puzzling that the BDZ receptor agonists considered here prolonged mIPSCs (Fig. 2), while deactivation of currents elicited by 100 μM and higher [GABA] was not affected. Although we have no definite explanation for these discrepancies, we would like to propose a mechanism that appears plausible and is compatible with all our experimental findings. The fact that the major effects of BDZs on amplitude and on deactivation kinetics are observed at GABA concentration markedly lower than the EC_{50} value, suggests that they require conditions in which a considerable percentage of receptors are singly bound. Macdonald *et al.* (1989) have proposed that, at micromolar [GABA], a considerable proportion of channel activity is due to singly bound $GABA_A$ receptors. The increase in binding affinity by BDZ would increase the percentage of doubly bound receptors. This, in turn, would alter the deactivation kinetics because the rate constants of opening/closing and desensitization are different in doubly and singly bound receptors. This prediction is, to a smaller or larger extent, reproduced by each model in which singly bound open states are postulated (e.g. Macdonald *et al.* 1989; Jones & Westbrook, 1995). Such a mechanism predicts that at higher [GABA], at which fully bound $GABA_ARs$ are predominant, BDZ impact on deactivation would be smaller. However, a clear effect of BDZs on mIPSCs

decay kinetics (Fig. 2) remains puzzling, as synaptic [GABA] certainly exceeds micromolar concentrations. Assuming that the synaptic agonist efficiency is reasonably described by the product of peak concentration and duration of agonist exposure (Barberis *et al.* 2004), the action of synaptic GABA transient would be comparable to application of hundreds of micromoles for ∼1 ms. However, our experiments show that at 100 μM GABA, BDZs did not affect deactivation either for neuronal or recombinant $\alpha1\beta2\gamma2$ receptors. An alternative possibility is that synaptically released agonist spills over from the cleft and activates perisynaptic $GABA_A$Rs. Clearly, GABA spilling over from a synapse reaches perisynaptic receptors at concentrations lower than in the cleft and therefore the ensuing current is more susceptible to modulation by BDZs. The enhancement of this current would appear as an up-regulation of the 'slow' IPSC component because of prolonged diffusion and slower kinetics due to low [GABA]. A similar proposal that IPSCs are partially shaped by subsaturating [GABA] has been put forward by Hill *et al.* (1998) who studied modulation of GABAergic synaptic currents by diethyl-lactam that affected currents evoked by non-saturating [GABA] but had no effect on responses to saturating [GABA]. There is a general agreement that the phenomenon of GABA spill-over may have a pronounced impact on GABAergic currents, especially in the case of intense synaptic activity (e.g. Isaacson *et al.* 1993; Overstreet & Westbrook, 2003) although blockade of GABA uptake exerts only a weak, if any, effect on mIPSCs. Interestingly, Hill reported that the contribution of subsaturating [GABA] to IPSCs was insensitive to blockade of the GABA uptake system. Altogether, we propose that the major mechanisms of mIPSCs modulation by BDZs are related to: (i) enhancement of the binding rate that has a particularly strong effect in conditions of non-saturation and fast agonist transient, and (ii) enhancement of current evoked by low [GABA] spilling over from the synapse that is manifested as a slow down of mIPSC. Although the latter proposal is compatible with our observations, we have no direct evidence for it and therefore, at the present stage, it remains speculative. Moreover, kinetic behaviour of synaptic and extrasynaptic $GABA_A$ receptors (even of the same type) might differ because of possible modulatory post-translational processes mediated by, for example, phosphorylation/dephosphorylation or binding to regulatory proteins (e.g. GABARAP or gephyrine).

Contrary to our findings, Mellor & Randall (1997) and Krampfl *et al.* (1998) reported that BDZs prolonged deactivation kinetics of currents evoked by high [GABA]. The reason for this discrepancy is not clear. However, in these studies the fast deactivation component was at least one order of magnitude slower than that reported here. This may suggest that in the protocols applied by Mellor & Randall (1997) and Krampfl *et al.* (1998) the fast component was not detected. Other factors, such as differences in benzodiazepine type, cellular model or GABA application protocols could also underlie these different observations.

The observation that BDZ receptor agonists might affect different $GABA_A$R properties (binding and gating) is not surprising. Walters *et al.* (2000) provided evidence for two functionally distinct BDZ binding sites on $\alpha1\beta2\gamma2$ receptors. It is thus possible that different sensitivities to flurazepam of currents evoked by 1 μM GABA (Fig. 3) and on deactivation of currents evoked by saturating [GABA] (a significant effect only at 10 μM, Fig. 4) might involve the presence of different BDZ binding sites.

An important conclusion of this work is that the proposed mechanisms of BDZ receptor agonist action implicates them as potent modulators of both tonic and phasic GABAergic currents. Qualitatively, their effect on the tonic component appears larger, which seems important as it is believed that tonic currents mediate considerably larger charge transfer than the phasic ones (Farrant & Nusser, 2005). On the other hand, it needs to be born in mind that the tonic and phasic forms of inhibition are functionally coupled. Indeed, ambient [GABA] depends on network excitability while shunting (tonic) GABAergic conductance has a major impact on the neuronal firing and therefore on synaptic signalling.

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