Postsynaptically different inhibitory postsynaptic currents in Cajal–Retzius cells in the developing neocortex

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Fast and slowly rising inhibitory postsynaptic currents (IPSCs, $IPSC_F$ and $IPSC_S$) in neocortical Cajal–Retzius cells are observed. In this study, zolpidem, a benzodiazepine agonist that specifically modulates γ-aminobutyric acid type A receptors ($GABA_A$Rs) containing γ_2 subunit, was used to characterize $GABA_A$Rs mediating $IPSC_F$ and $IPSC_S$. One-hundred-nanomolar zolpidem prolonged $IPSC_S$, increased evoked $IPSC_S$ ($eIPSC_S$) amplitude, and decreased paired-pulse ratio (PPR) of $eIPSC_S$. Two micromolar zolpidem prolonged both $IPSC_F$ and $IPSC_S$, increased miniature $IPSC_F$ and $eIPSC_F$ amplitudes, increased $eIPSC_S$ amplitude but not miniature $IPSC_S$ amplitude, decreased PPR of $eIPSC_S$, but failed to affect PPR of $eIPSC_F$. We conclude that $IPSC_F$ are mediated by $\alpha_{2/3}$-containing $GABA_A$Rs, which are not saturated by synaptic GABA release, whereas $IPSC_S$ are mediated by α_1-containing and $\alpha_{2/3}$-containing $GABA_A$Rs, which are saturated by quantal GABA release. *NeuroReport* 19:1213–1216 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Cajal–Retzius (CR) cells are the principal neurons in the marginal zone/layer I of the developing neocortex. These early-born neurons have large pericarya with long horizontal dendrites and axonal arbors that are restricted to layer I. CR cells represent a transient cell population and disappear approximately by the end of the second postnatal week in rodents [1,2]. Owing to their strategic location in layer I and the coincidence of their life span with the period of cortical migration, CR cells have been proposed to play a key role in the structural organization of the neocortex. Recent experimental findings have shown that CR cells synthesize and secrete reelin, an extracellular matrix protein necessary for cortical lamination [3]. In reelin-deficient mice the inside-out formation of the neocortex is compromised [4].

CR cells receive excitatory γ-aminobutyric acid (GABA)ergic inputs [5,6] and are involved in the synchronized network activity in layer I in both wild-type and reelin-deficient mice [7]. Recently, we have shown that CR cells receive two types of GABAergic inputs [8]. As CR cells express γ_2 subunit-containing $GABA_A$ receptors ($GABA_A$Rs) during postnatal development [9], in this study we have used zolpidem, a benzodiazepine agonist that specifically modulates $GABA_A$Rs containing γ_2 subunit. Moreover, zolpidem modulates $\alpha_1\gamma_2$-containing, $\alpha_{2/3}\gamma_2$-containing, and $\alpha_5\gamma_2$-containing $GABA_A$Rs with K_i values of 20, 450, and 15 000 nM [10]. Therefore, different zolpidem concentrations were used to assess the composition of postsynaptic $GABA_A$Rs and to investigate whether $GABA_A$Rs are saturated by the synaptically released GABA [11].

Materials and methods

Preparation of brain slices

All experiments were conducted with pigmented C57BL/6J mice pups of postnatal days (P) 5–7 (the day of birth was designated as P0). Animals were decapitated under deep ether anesthesia. The brain was removed quickly and transferred into ice-cold saline that contained (in mM): 125 NaCl, 4 KCl, 10 glucose, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 0.5 $CaCl_2$, and 2.5 $MgCl_2$ constantly aerated with a 5% CO_2–95% O_2 mixture (pH=7.3). Sagittal slices of both hemispheres were cut on a vibratome (Campden Instruments Ltd, Loughborough, Leicestershire, UK). After preparation, slices (200-μm-thick) were stored for at least 1 h at room temperature in artificial cerebrospinal fluid (ACSF) that contained (in mM): 125 NaCl, 4 KCl, 10 glucose, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 2 $CaCl_2$, and 1 $MgCl_2$. pH was buffered to 7.3 by continuous bubbling with a 5% CO_2–95% O_2 mixture. All experiments were carried out according to the guidelines laid down by the Landesamt für Arbeitsschutz, Gesundheitsschutz und technische Sicherheit Berlin, T0406/03.

Electrophysiological recordings in acute slices

For recordings, slices were placed into a recording chamber (~0.4 ml volume) on the microscope stage (Axioscope FS,

Zeiss, Oberkochen, Germany) equipped with phase contrast optics. Slices were submerged with a constant flow of oxygenated ACSF. Flow rate was set to 1 ml/min. A 40x water immersion objective was used in all experiments. CR cells were visually selected according to morphological criteria: (i) location in layer I, (ii) horizontal orientation, (iii) large ovoid soma, and (iv) one thick-tapered dendrite typically extended in parallel to the pial surface.

Ten micromolar 6,7-dinitroquinoxaline-2,3-dione (an AMPA/kainate receptor antagonist) and 50 μM DL-2-amino-5-phosphonopentanoic acid (a *N*-methyl D-aspartate receptor blocker) were added to the ACSF to block glutamatergic currents. Inhibitory postsynaptic currents (IPSCs) were recorded using the whole-cell configuration of the patch-clamp technique. Intrapipette solution contained (in mM): 100 potassium gluconate, 50 KCl, 5 NaCl, 0.5 $CaCl_2$, 5 ethylene glycol-bis (*b*-aminoethyl ether), 25 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 2 MgATP, 0.3 GTP, pH was set to 7.2 with KOH. Pipette resistance was 3–5 MΩ, when filled with the above saline. Electrophysiological signals were acquired using an EPC-7 amplifier (List Electronics, Darmstadt, Germany), a 16-bit AD/DA board (ITC-16, HEKA Elektronik, Lambrecht, Germany), and TIDA 4.11 software (HEKA Elektronik). The signals were filtered at 3 kHz and sampled at a rate of 10 kHz. Access resistance was controlled by applying hyperpolarizing pulses of 10 mV. Only recordings with a series resistance below 40 MΩ were accepted. Series resistance compensation was not applied. Cells exhibiting more than 20% changes in the access resistance during an experiment were discarded. The chloride reversal potential was about −20 mV. The holding potential was set to −70 mV. In this study, $GABA_A$R-mediated postsynaptic currents will be referred to as IPSCs even though the action of GABA is depolarizing in CR cells.

Electrical stimulation

Evoked postsynaptic currents were elicited by focal electrical stimulation through a glass pipette filled with ACSF (about 10 MΩ). In this case, quinoxaline 314 (2 mM) was added to the intracellular solution to prevent generation of action potentials in the tested neurons. An isolated stimulation unit was used to generate rectangular electrical pulses. Pulse duration was set to 0.5 ms. Pulse intensity was adjusted to activate a unitary synaptic input (minimal stimulation). Simulation was accepted as minimal if the following criteria were satisfied: (i) evoked inhibitory postsynaptic current (eIPSC) latency remained stable (<20% fluctuations); (ii) lowering stimulus intensity by 20% resulted in a complete failure of eIPSCs; and (iii) an increase in stimulus intensity by 20% changed neither mean eIPSC amplitude nor eIPSC shape. Typical pulse intensity required for minimal stimulation was between 1 and 2 μA.

Miniature inhibitory postsynaptic current and evoked inhibitory postsynaptic current separation

Recently, we have shown that in CR cells the distribution of miniature inhibitory postsynaptic current (mIPSC) rise times is better fitted with the sum of two Gaussian functions [8]. Therefore, we separated fast and slowly rising mIPSCs ($mIPSC_F$ and $mIPSC_S$) using the following procedure. mIPSC rise time distribution was fitted by two Gaussians. After this, all mIPSCs with rise times shorter than the first peak plus two standard deviations were selected as $mIPSC_F$. All mIPSCs with longer rise times were defined as $mIPSC_S$. eIPSCs with rise times shorter and longer than 1 ms were defined as $eIPSC_F$ (typical value was 0.7 ms) and $eIPSC_S$ (typically 1.3 ms), respectively.

Superfusion

All experiments were carried out at room temperature (22–25°C). Tetrodotoxin was obtained from Alomone Labs (Jerusalem, Israel). All other chemicals were obtained from Sigma-Aldrich (Munich, Germany).

Data evaluation and statistics

Data were evaluated off-line using PeakCount V3.2 software (C. Henneberger, Institute of Neurophysiology, Berlin, Germany). The program uses a derivative threshold-crossing algorithm to detect individual IPSCs. Each automatically detected event is displayed for visual inspection. IPSC rise (10–90%) and half-decay times can be also obtained. All results are presented as mean ± SEM. The error bars in all figures indicate SEM. Means were tested for significance using one population Student's *t*-test.

Results

Zolpidem differentially modulates fast rising miniature inhibitory postsynaptic current and slowly rising miniature inhibitory postsynaptic current

As extracellular [GABA] in layer I of the developing cortex is relatively high [8], we asked if zolpidem affects the resting membrane conductance. In all cases, zolpidem changed neither the resting potential, nor the membrane resistance, nor the holding current (n=13, data not shown) of CR cells.

Next, we asked if zolpidem affects mIPSCs. mIPSCs were recorded in the presence of 1 μM tetrodotoxin. In addition, to increase mIPSC frequency, *N*-ethylmaleimide (50 μM) was preapplied for 5 min. *N*-ethylmaleimide was shown to affect neither mIPSC amplitudes nor kinetics in CR cells [12]. One-hundred nanomolar zolpidem, a concentration at which the drug modulates only α_1-containing $GABA_A$Rs, influenced neither the median amplitude of $mIPSC_F$ (107 ± 9% of control, $P>0.3$) nor $mIPSC_F$ half-decay time (109 ± 12% of control, $P>0.2$, n=18, Fig. 1b). In contrast to $mIPSC_F$, $mIPSC_S$ half-decay time was significantly increased (131 ± 6% of control, $P<0.001$), whereas the median amplitude of $mIPSC_S$ was unaffected (104 ± 6% of control, $P>0.4$, n=18, Fig. 1b) by 100 nM zolpidem.

Two micromolar zolpidem, a concentration at which zolpidem also affects $\alpha_{2/3}$-containing $GABA_A$Rs, increased the median amplitude of $mIPSC_F$ to 126 ± 5% of control ($P<0.001$, n=14) and $mIPSC_F$ half-decay time to 166 ± 8% of control ($P<0.001$, n=14, Fig. 1a, B). The median amplitude of $mIPSC_S$ was not changed (108 ± 9% of control, $P>0.3$), whereas $mIPSC_S$ half-decay time was further increased (173 ± 10% of control, $P<0.001$, n=14, Fig. 1a and b). Interestingly, 2 μM zolpidem decreased the coefficient of variation [coefficient of variation (CV)=the mean mIPSC amplitude divided by the standard deviation] of $mIPSC_S$ (88 ± 3% of control, $P<0.05$, n=14, Fig. 1b). Ten micromolar zolpidem applied after 2 μM zolpidem failed to produce any further effect on both $mIPSC_F$ and $mIPSC_S$ (n=5, data not shown). Thus, $mIPSC_F$ seems to be mediated mainly by $\alpha_{2/3}$-containing $GABA_A$Rs, whereas both $\alpha_{2/3}$-containing and α_1-containing $GABA_A$Rs contribute to $mIPSC_S$ generation. Moreover, as 2 μM zolpidem increases the median amplitude

of $mIPSC_F$, postsynaptic $GABA_A$Rs seem to be not saturated by the quantal GABA release at synapses that generate these currents.

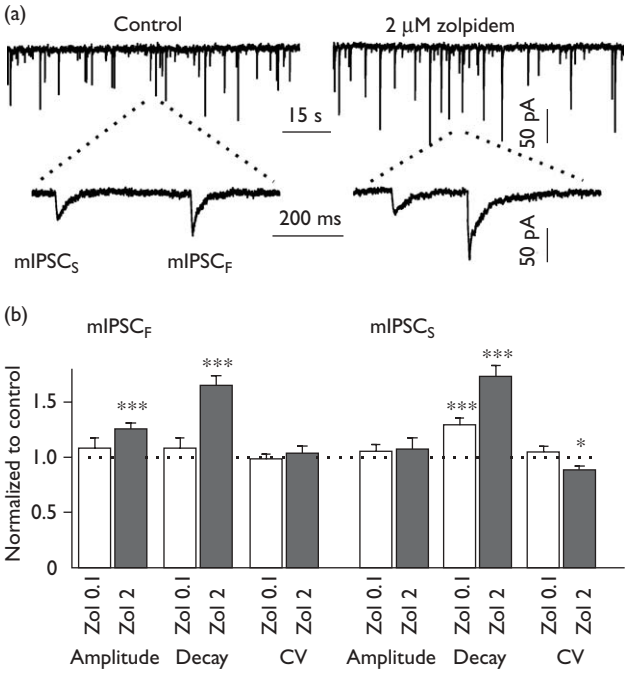

Fig. 1 Zolpidem (Zol) effects on mIPSCs. (a) Sample traces demonstrate mIPSCs recorded in control and in the presence of 2 μM Zol. Insets show $mIPSC_F$ and $mIPSC_S$. (b) Statistical data showing the effects of Zol (0.1 μM – open bars, 2 μM – grey bars) on the median amplitude, half-decay time and CV of $mIPSC_F$ (left) and $mIPSC_S$ (right). $^*P<0.05$, $^{***}P<0.001$. CV, coefficient of variation; mIPSC, miniature inhibitory postsynaptic current; $mIPSC_F$, fast rising miniature inhibitory postsynaptic current; $mIPSC_S$, slowly rising miniature inhibitory postsynaptic current.

Fast rising evoked inhibitory postsynaptic current and slowly rising evoked inhibitory postsynaptic current differ in their sensitivity to zolpidem

To corroborate these results, we investigated zolpidem effects on eIPSCs. eIPSCs were elicited using a paired-pulse protocol with interstimulus interval of 50 ms. Similar to the zolpidem effects on $mIPSC_F$, only 2 μM zolpidem affected the mean amplitude of $eIPSC_F$ (126±9% of control, $P<0.05$) and $eIPSC_F$ half-decay time (167±10% of control, $P<0.001$, $n=9$, Fig. 2a and b). Similar to $mIPSC_S$, $eIPSC_S$ half-decay time was increased by zolpidem [126±6% ($P<0.01$, $n=10$) and 167±7% of control ($P<0.001$, $n=8$) in the presence of 100 nM and 2 μM zolpidem, respectively]. Surprisingly, zolpidem increased the mean amplitude of $eIPSC_S$ [110±4% ($P<0.05$, $n=10$) and 128±8 of control ($P<0.01$, $n=8$] in the presence of 100 nM and 2 μM of zolpidem, respectively, Fig. 2c and d). Moreover, zolpidem decreased the paired-pulse ratio of $eIPSC_S$ [90±3% ($P<0.05$, $n=10$) and 72±8% of control ($P<0.01$, $n=8$) in the presence of 100 nM and 2 μM zolpidem, respectively], but not of $eIPSC_F$ [98±11% ($P>0.9$, $n=9$) and 98±7% ($P>0.87$, $n=9$) in the presence of 100 nM and 2 μM zolpidem, respectively, Fig. 2b and d].

Discussion

Neocortical CR cells have been shown to receive two GABAergic inputs, which generate $IPSC_F$ and $IPSC_S$. $IPSC_F$ projections have lower release probability and are capable to transfer information at high frequency, whereas $IPSC_S$ connections already become ineffective at 1 Hz [8]. In this study, we investigated if these synapses differ postsynaptically. As postnatal CR cells express only γ-containing $GABA_A$Rs [9], the benzodiazepine agonist zolpidem has been used. Zolpidem increases the affinity of $\alpha_1\gamma_2$-, $\alpha_{2/3}\gamma_2$-, and $\alpha_5\gamma_2$-containing $GABA_A$Rs for GABA with K_i values of 20, 450, and 15 000 nM [10]. Therefore, to unravel an

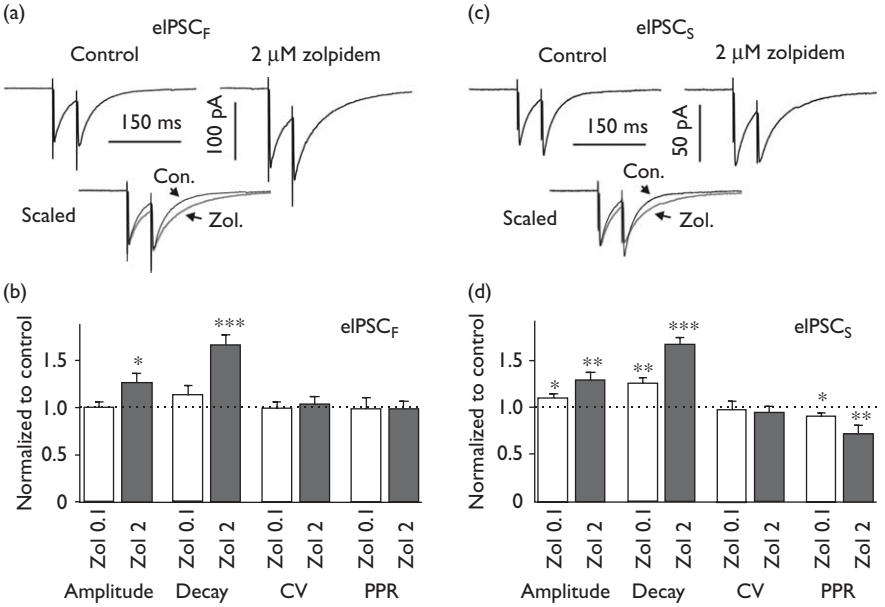

Fig. 2 Zolpidem (Zol) effects on eIPSCs. (a, c). Traces show $eIPSC_F$ (a) and $eIPSC_S$ (c) elicited by paired-pulse protocol in control and in the presence of 2 μM Zol. Traces are an average of 40 trials. Insets: eIPSCs in control and in the presence of Zol were scaled to demonstrate the effects of Zol on eIPSC kinetics and PPR. (b, d). Statistical data demonstrating the effects of Zol (0.1 μM – open bars, 2 μM – grey bars) on $eIPSC_F$ (b) and $eIPSC_S$ (d). $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. Con., control; eIPSC, evoked inhibitory postsynaptic current; $eIPSC_F$, fast rising evoked inhibitory postsynaptic current; $eIPSC_S$, slowly rising evoked inhibitory postsynaptic current; PPR, paired-pulse ratio.

involvement of $\alpha_1\gamma_2$-subunits, we applied 100 nM zolpidem. One-hundred nanomolar zolpidem prolonged selectively $IPSC_S$ suggesting that $\alpha_1\gamma_2$-containing $GABA_A$Rs contribute to $IPSC_S$, but not $IPSC_F$ generation. As 2 μM zolpidem increased the duration of both $IPSC_F$ and $IPSC_S$ to a similar extent (170% of control), $\alpha_{2/3}\gamma_2$-containing $GABA_A$Rs seem to mediate both types of IPSCs.

The zolpidem-induced increase in $GABA_A$R affinity allows assessing the degree of $GABA_A$R occupancy. If $GABA_A$Rs are saturated by the synaptically released GABA, zolpidem should minimally influence mIPSC amplitude. Indeed, the median $mIPSC_S$ amplitude was not changed by zolpidem. In contrast to $mIPSC_S$, $mIPSC_F$ amplitudes increased to 126% of control in the presence of 2 μM zolpidem. As $\alpha_1\gamma_2$-containing $GABA_A$Rs contribute only to $IPSC_S$, distinct $GABA_A$R affinities for GABA at $IPSC_F$ and $IPSC_S$ synapses may underlie the observed effects. Lower affinity receptors should, however, produce more rapidly decaying mIPSCs [13], but this was not observed. $mIPSC_F$ and $mIPSC_S$ demonstrated similar half-decay times (10.5 and 10.8 ms) in control and were prolonged to a similar extent (170% of control) by 2 μM zolpidem. Thus, it is unlikely that $GABA_A$R affinity is the major determinant of the degree of $GABA_A$R occupancy.

Two other possibilities are difficult to separate. First, different numbers of postsynaptic $GABA_A$Rs may be located at $IPSC_F$ and $IPSC_S$ synapses. Correlation between mIPSC amplitude and postsynaptic receptor number has been directly demonstrated in cerebellar stellate cells. Moreover, the benzodiazepine agonist flurazepam prolonged all mIPSCs, but selectively increased the amplitude of large events [14]. In CR cells the median amplitude of $mIPSC_F$ (~55 pA) is about twice that of $mIPSC_S$ (~25 pA) suggesting that a larger $GABA_A$R number at $IPSC_F$ synapses may underlie their incomplete occupancy by synaptically released GABA. Second, [GABA] or the duration of GABA transients in the synaptic cleft may determine the degree of $GABA_A$R occupancy [15]. If postsynaptic $GABA_A$Rs are not saturated by GABA and the variance of mIPSC amplitudes solely results from fluctuations of [GABA] in the synaptic cleft, an increase in $GABA_A$R affinity should reduce quantal variance. The CVs of $mIPSC_F$ (0.62 vs. 0.63) as well as $eIPSC_F$ (0.69 vs. 0.68 in controls and in the presence of 2 μM zolpidem, respectively) were not significantly changed by zolpidem. Thus, incomplete $GABA_A$R occupancy is probably due to a larger number of postsynaptic receptors located at these synapses.

Interestingly, the CV of $mIPSC_S$ decreased in the presence of 2 μM zolpidem (0.47 and 0.41 in control and in 2 μM zolpidem, respectively). Zolpidem-induced increase in the open probability of $GABA_A$Rs may result in the reduction of $mIPSC_S$ CV even when $GABA_A$Rs are fully occupied by synaptically released GABA. Indeed, $eIPSC_S$ amplitude was augmented by zolpidem. The CV of $eIPSC_S$ was, however, not decreased by zolpidem arguing against the suggestion. The zolpidem-induced increase of $eIPSC_S$ amplitude could be mediated by presynaptic $GABA_A$Rs. This is, however, unlikely because zolpidem (i) failed to increase mIPSC frequency and (ii) did not affect the failure rate of $eIPSC_S$. Alternatively, GABA diffusion from the synaptic cleft and activation of extrasynaptic $\alpha_{2/3}\gamma_2$-containing $GABA_A$Rs can underlie the observation. GABA diffusion is definitely not affected by zolpidem. Therefore, zolpidem-induced increase in $GABA_A$R affinity, that is, an increase in the number of $GABA_A$Rs activated by GABA in response to the first stimulus, should decrease the number of receptors available for the second release. Indeed, zolpidem decreased paired-pulse ratio of $eIPSC_S$. We suggest that $IPSC_S$ synapses (presumably subplate-layer I projections [8]) are characterized by smaller number of $GABA_A$Rs and weaker GABA clearance as compared with $IPSC_F$ contacts (presumably GABAergic thalamocortical projections [16]). Thus, the information transfer at $IPSC_S$ connections is limited both presynaptically (high-release probability) and postsynaptically (saturation of $GABA_A$Rs), whereas low-release probability and incomplete occupancy of $GABA_A$Rs at $IPSC_F$ projections make them capable to operate at higher rate.

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