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Loss of zolpidem efficacy in the hippocampus of mice with the $GABA_A$ receptor $\gamma2$ F77I point mutation

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Abstract

Zolpidem is a hypnotic benzodiazepine site agonist with some γ-aminobutyric acid $(GABA)_A$ receptor subtype selectivity. Here, we have tested the effects of zolpidem on the hippocampus of $\gamma2$ subunit ($\gamma2$F77I) point mutant mice. Analysis of forebrain $GABA_A$ receptor expression with immunocytochemistry, quantitative [^{3}H]muscimol and [^{35}S] t-butylbicyclophosphorothionate (TBPS) autoradiography, membrane binding with [^{3}H]flunitrazepam and [^{3}H]muscimol, and comparison of miniature inhibitory postsynaptic current (mIPSC) parameters did not reveal any differences between homozygous $\gamma2$I77/I77 and $\gamma2$F77/F77 mice. However, quantitative immunoblot analysis of $\gamma2$I77/I77 hippocampi showed some increased levels of $\gamma2$, $\alpha1$, $\alpha4$ and δ subunits, suggesting that differences between strains may exist in unassembled subunit levels, but not in assembled receptors. Zolpidem (1 μM) enhanced the decay of mIPSCs in CA1 pyramidal cells of control (C57BL/6J, $\gamma2$F77/F77) mice by ~60%, and peak amplitude by ~20% at 33–34 °C *in vitro*. The actions of zolpidem (100 nM or 1 μM) were substantially reduced in $\gamma2$I77/I77 mice, although residual effects included a 9% increase in decay and 5% decrease in peak amplitude. Similar results were observed in CA1 stratum oriens/alveus interneurons. At network level, the effect of zolpidem (10 μM) on carbachol-induced oscillations in the CA3 area of $\gamma2$I77/I77 mice was significantly different compared with controls. Thus, the $\gamma2$F77I point mutation virtually abolished the actions of zolpidem on $GABA_A$ receptors in the hippocampus. However, some residual effects of zolpidem may involve receptors that do not contain the $\gamma2$ subunit.

Introduction

γ-Aminobutyric acid $(GABA)_A$ receptors are pentameric ligand-gated anion channels comprised of diverse subunits ($\alpha1$–6, $\beta1$–3, $\gamma1$–3, δ, ε, θ, π, $\rho1$–3) that are heterogeneously distributed throughout the brain (Wisden *et al*., 1992; Pirker *et al*., 2000; Sieghart & Sperk, 2002). Neurons can contain numerous subunit species, enabling the potential expression of many receptor subtypes, each with characteristic properties, including sensitivity to allosteric modulators (Sieghart, 1995; Whiting *et al*., 2000; Korpi *et al*., 2002a). Rat pyramidal cells of the hippocampal CA1 area express up to 14 $GABA_A$ receptor subunits (Persohn *et al*., 1992; Wisden *et al*., 1992; Fritschy & Mohler, 1995; Sperk *et al*., 1997; Wegelius *et al*., 1998; Ogurusu *et al*., 1999). The GABAergic inputs of pyramidal cells arise from heterogeneous interneuron populations that preferentially target either the dendrites, soma or axon initial segment of the pyramidal cells (Freund & Buzsáki, 1996; Maccaferri & Lacaille, 2003; Somogyi & Klausberger, 2005). Interneurons phase the rhythmic activity of pyramidal neurons (Klausberger *et al*., 2003) during hippocampal-related behaviour (Csicsvari *et al*., 1999). Some $GABA_A$ subtypes, containing distinct receptor subunits, are targeted to synapses innervated by specific interneuron types (Nusser *et al*., 1996; Nyíri *et al*., 2001; Klausberger *et al*., 2002), and inhibitory postsynaptic potentials (IPSPs) generated by distinct interneuron populations are differentially modulated by allosteric ligands (Pawelzik *et al*., 1999; Thomson *et al*., 2000). In addition, different populations of interneurons may express specific receptor subunits and therefore also be differentially modulated by allosteric ligands (Gao & Fritschy, 1994; Brünig *et al*., 2002).

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Zolpidem is a widely used hypnotic that binds to the benzodiazepine (BZ) site of the $GABA_A$ receptor formed at the junction of α and γ subunits (Sigel, 2002; Ernst *et al*., 2003). The α1 subunit-containing receptors show increased sensitivity to zolpidem (Pritchett & Seeburg, 1990; Crestani *et al*., 2000), resulting in allosteric potentiation of the actions of GABA. This can be detected as an increase in the decay, and sometimes the amplitude, of miniature inhibitory postsynaptic currents (mIPSCs) (De Koninck & Mody, 1994; Perrais & Ropert, 1999; Hájos *et al*., 2000; Patenaude *et al*., 2001; Goldstein *et al*., 2002).

As determined in α1βγ2 recombinant receptors, the binding of zolpidem to the BZ site requires phenylalanine (F) at position 77 in the γ2 subunit. In both heterologous expression systems and in mice, substitution of this residue with an isoleucine (I) produces zolpidem-insensitive receptors (Buhr *et al*., 1997; Wingrove *et al*., 1997; Cope *et al*., 2004; Ogris *et al*., 2004). Previously we have shown that zolpidem sensitivity of mIPSCs in cerebellar Purkinje cells of mice with the γ2F77I point mutation is eliminated (Cope *et al*., 2004). Furthermore, the effects of zolpidem on the rotarod test, which has a known cerebellar contribution, were completely abolished in γ2F77I mice compared with control mice. Purkinje cells express few receptor subunits and form a single receptor subtype, α1β2/3γ2 (e.g. Wisden *et al*., 1996). These experiments did not exclude the possibility that zolpidem remained an effective $GABA_A$ receptor modulator in neurons expressing a multitude of $GABA_A$ receptors. Here we have tested the effects of the γ2F77I point mutation in hippocampal CA1 pyramidal cells and GABAergic interneurons. Some of these data have been previously reported as an abstract (Halbsguth *et al*., 2004).

Materials and methods

Generation and genotyping of the γ2F77I point mutant mice

The F77I point mutation in the $GABA_A$ receptor γ2 subunit was generated by homologous recombination in 129ola embryonic stem cells (Cope *et al*., 2004). Heterozygous γ2F77/I77 breeding pairs were crossed to give homozygous γ2I77/I77 point mutant mice and γ2F77/F77 littermate controls that were used in experiments. Mice were genotyped by polymerase chain reaction according to the details given in Cope *et al*. (2004). In addition, because C57BL/6J mice (Charles River Deutschland, Sulzfeld, Germany) were used to produce the F1 generation, we also used these mice for further comparisons in some experiments.

Immunocytochemistry of perfusion-fixed hippocampi

Three adult γ2I77/I77 homozygous mice (25–30 g) and three littermate γ2F77/F77 homozygous mice (25–30 g) were used for immunocytochemical procedures. Mice were deeply anaesthetized with sodium pentobarbital (100 mg/kg, i.p.) and transcardially perfused in accordance with the UK Animals (Scientific Procedure) Act 1986 and associated procedures. The initial solution was 0.1 M phosphate-buffered saline (PBS), followed for 7 min by a fixative composed of 4% paraformaldehyde and ~0.2% picric acid made up in 0.1 M phosphate buffer (PB, pH 7.2). Brains were quickly removed, extensively rinsed in PB and sectioned in the sagittal plane at 50 μm thickness on a vibratome. Immunocytochemical reactions were performed according to the indirect avidin–biotin–horseradish peroxidase (HRP) complex procedure (Vectastain ABC Elite kit, Vector Burlingame, CA, USA), as described previously (Cope *et al*., 2004). Affinity-purified antibodies for the α1 (0.6 μg/mL), β3 (1 μg/mL) and γ2 (1 μg/mL) subunits were the same as used for the immunoblot experiments. In addition, affinity-purified guinea pig antibodies to the α2 subunit (1.5 μg/mL, residues 1–9, Fritschy & Mohler, 1995) and to the α5 subunit (1 : 500, residues 1–10, Fritschy & Mohler, 1995) were used. For antibody specificity see papers quoted in the immunoblotting section, and for distribution maps in the brain see Pirker *et al*. (2000). Peroxidase enzyme activity was revealed using 3,3′-diaminobenzidine tetrahydrochloride (0.5 mg/mL in 50 mM Tris–HCl buffer, pH 7.4; Sigma, UK) as chromogen and 0.003% H_2O_2 as substrate. The duration of the enzyme reaction was between 5 and 10 min.

Quantitative immunoblot analysis of $GABA_A$ receptor subunits

Hippocampi from adult γ2F77/F77 or γ2I77/I77 mice were individually homogenized using an Ultra-Turrax® in 50 mM Tris/citrate buffer (pH 7.1) containing one complete protease inhibitor cocktail tablet per 50 mL buffer (Roche Diagnostics, Mannheim, Germany). Equal amounts (containing 7 μg of protein) of the suspension were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis in different slots of the same 10% polyacrylamide gel. Proteins were blotted to polyvinylidene difluoride membranes and detected by antibodies to the following subunits: α1 (amino acid residues 1–9); α2 (322–357); α4 (379–421); β2 (351–405); β3 (345–408); γ2 (319–366); and δ (1–44) (Jechlinger *et al*., 1998; Pöltl *et al*., 2003). Secondary antibodies [$F(ab')_2$ fragments of goat anti-rabbit IgG, coupled to alkaline phosphatase, Axell, Westbury, NY, USA] were visualized by the reaction of alkaline phosphatase with CDP-Star (Applied Biosystems, Bedford, MA, USA). The chemiluminescent signal was quantified by densitometry after exposing the immunoblots to the Fluor-S MultiImager (Bio-Rad Laboratories, Hercules, CA, USA) and evaluated using Quantity One Quantification Software (Bio-Rad Laboratories) and GraphPad Prism (GraphPad Software, San Diego, CA, USA). Quantification was performed by an independent investigator blind to the identity of the samples. Immunoreactivities were within the linear range, as established by measuring the antibody-generated signal to a range of antigen concentrations, permitting a direct comparison of the amount of antigen per gel lane between samples. Data were generated from three different gels per subunit per mouse, and are expressed as mean ± SE. Student's unpaired *t*-test was used for comparing groups, and significance was set at $P < 0.05$.

Autoradiography of [^{3}H]muscimol and [^{35}S] t-butylbicyclophosphorothionate (TBPS) binding

Adult mice were killed by decapitation, and whole brains were rapidly dissected out and frozen on dry ice. Coronal cryostat sections (14 μm) were cut from five γ2I77/I77 and five γ2F77/F77 mouse brains, thaw-mounted onto gelatine-coated object glasses, and stored frozen under desiccant at −20 °C. All experiments were carried out in parallel fashion with respect to mouse lines, eliminating any day-to-day variation between the assays. The autoradiographic procedures for regional localization of [^{3}H]Ro 15-4513, [^{3}H]muscimol and [^{35}S]TBPS binding were as described (Mäkelä *et al*., 1997). Briefly, sections were preincubated in an ice-water bath for 15 min in 50 mM Tris–HCl (pH 7.4) supplemented with 120 mM NaCl in the [^{3}H]Ro 15-4513 and [^{35}S]TBPS autoradiographic assays, and in 0.31 M Tris–citrate (pH 7.1) in the [^{3}H]muscimol assay. All radioligands were purchased from Perkin Elmer Life Sciences (Boston, MA, USA). The final incubation in respective preincubation buffer was performed with 6 nM [^{35}S]TBPS at room temperature for 90 min, assays with 10 nM [^{3}H]muscimol at 0–4 °C for 30 min, and assays with 10 nM [^{3}H]Ro

15-4513 at 0–4 °C for 60 min. After incubation, sections were washed 3 × 15 s or 2 × 30 s in an ice-cold incubation buffer in [^{35}S]TBPS and [^{3}H]Ro 15-4513 or in [^{3}H]muscimol assay, respectively. Sections were then dipped into distilled water, air-dried under a fan at room temperature, and exposed with plastic [^{3}H]- or [^{14}C]-methacrylate standards to Kodak Biomax MR films for 1–8 weeks. Non-specific binding determined with 10 μM flumazenil (Hoffmann-La Roche, Basel, Switzerland), 100 μM picrotoxin (Sigma) and 100 μM GABA (Sigma) in [^{3}H]Ro 15-4513, [^{35}S]TBPS and [^{3}H]muscimol assays, respectively, did not differ from the background. Hippocampal binding densities were quantified with MCID M5-imaging software (Imaging Research, Ontario, Canada) and converted to nCi/mg or nCi/g radioactivity values on the basis of the simultaneously exposed standards. Data are presented as mean ± SD.

Preparation of hippocampal membranes and [^{3}H]flunitrazepam receptor-binding studies

Hippocampi homogenated in Tris–citrate buffer (pH 7.1, see above) were ultracentrifuged at 150,000 ***g***. Pellets were washed three times in 10 mL and finally resuspended in 5 mL of the same buffer. For receptor-binding studies, 100 μL of the suspension was added to a final volume of 1 mL of a solution containing 50 mM Tris–citrate buffer (pH 7.1), 150 mM NaCl and 1–20 nM [^{3}H]flunitrazepam (84.5 Ci/mol, PerkinElmer Life Sciences) in the absence or presence of 10 μM diazepam. After incubation for 90 min at 4 °C, the suspensions were rapidly filtered through Whatman GF/B filters, washed twice with 5 mL of 50 mM Tris–citrate buffer (pH 7.1) and subjected to liquid scintillation counting (Filter-Count™, Packard; 2100 TR Tri-Carb® Scintillation Analyser, Packard). Binding in the presence of diazepam (unspecific binding) was then subtracted from binding in the absence of diazepam (total binding) to obtain specific binding to $GABA_A$ receptors. The experiment was performed independently four times and data were analysed using GraphPad Prism (GraphPad Software). Data are presented as mean ± SE and were compared using Student's unpaired *t*-test with significance set at $P < 0.05$.

Preparation of receptor extracts and [^{3}H]muscimol-binding studies

Hippocampi were homogenized using an Ultra-Turrax® in 5 mL of a deoxycholate buffer (0.5% deoxycholate, 0.05% phosphatidylcholine, 10 mM Tris–HCl and 150 mM NaCl, pH 8.5) containing one complete protease inhibitor cocktail tablet (Roche Diagnostics) per 50 mL. Homogenates were incubated under intensive stirring for 60 min at 4 °C and then ultracentrifuged at 150,000 ***g*** for 45 min. For the determination of the solubilization efficiency of immunoprecipitated receptors and subsequent [^{3}H]muscimol-binding studies, both the clear supernatant and redissolved pellet were used.

For immunoprecipitation, 150 μL protein of the clear supernatant as well as of the redissolved pellet were mixed with a solution containing 5 μg of α_1(1–9), 10 μg of β_1(350–404), 5 μg of β_2(351–405) and 5 μg of β_3(345–408) antibody in order to precipitate all $GABA_A$ receptors present in the hippocampal extract. This antibody composition was used because all functional $GABA_A$ receptors probably contain at least one of the three β subunits, and most of them contain an α_1 subunit (Jechlinger *et al.*, 1998; Tretter *et al.*, 2001). The mixture was then incubated at 4 °C overnight under gentle shaking. Then 50 μL of pansorbin (Calbiochem, La Jolla, CA, USA) and 50 μL 5% dry milk powder, both in low-salt buffer for immunoprecipitation (IP-low; 50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA and 0.2% Triton X-100, pH 8.0), were added and incubation was continued for 2 h at 4 °C under gentle shaking. The precipitate was centrifuged for 5 min at 2300 ***g***, washed twice with 500 μL of high-salt buffer for immunoprecipitation (IP-high; 50 mM Tris–HCl, 600 mM NaCl, 1 mM EDTA and 0.5% Triton X-100, pH 8.3) and once with 500 μL of IP-low. The precipitated receptors were suspended in 1 mL of a solution containing 0.1% Triton X-100, 50 mM Tris–citrate buffer (pH 7.1) and 1–40 nM [^{3}H]muscimol (29.5 Ci/mmol, Perkin-Elmer Life Sciences) in the absence or presence of 1 mM GABA. After incubation for 60 min at 4 °C the suspensions were rapidly filtered through Whatman GF/B filters, washed twice with 3.5 mL of 50 mM Tris–citrate buffer (pH 7.1) and subjected to liquid scintillation counting as above. Binding in the presence of 1 mM GABA (unspecific binding) was subtracted from binding in the absence of GABA (total binding), resulting in specific binding to precipitated $GABA_A$ receptors. The experiment was performed independently three times using hippocampi from different γ2F77/F77 and γ2I77/I77 mice. Data were analysed using GraphPad Prism (Graph Pad Software) and are presented as mean ± SE. Student's unpaired *t*-test was used for comparison between genotypes, and significance was set at $P < 0.05$.

Slice preparation and whole-cell patch-clamp electrophysiological recordings

Hippocampal slices were obtained from male C57BL/6J [postnatal day (P) 17–29; Charles River Laboratories, Margate, UK], and male and female γ2I77/I77 and γ2F77/F77 (P17–38 and P16–39, respectively) mice. Experiments on littermates of γ2I77/I77 and γ2F77/F77 mice from heterozygous breeding pairs were performed blind with regards to genotype. Briefly, mice were anaesthetized with isoflurane and decapitated, in accordance with the UK Animals (Scientific Procedures) Act 1986. The brains were rapidly removed and 300–350-μm-thick whole brain coronal or horizontal slices were cut in ice-cold artificial cerebrospinal (aCSF) of composition (in mM): NaCl, 126; $NaHCO_3$, 26; KCl, 2.5; $CaCl_2$, 2; $MgCl_2$, 2; NaH_2PO_4, 1.25; glucose, 10; and kynurenic acid, 3; final pH 7.3–7.4 when continuously oxygenated (95% O_2: 5% CO_2), adjusted with NaOH. Slices were stored for at least 1 h at room temperature in an incubation chamber containing the above continuously oxygenated aCSF, but without the kynurenic acid. Slices were perfused in the recording chamber with warmed (33–34 °C, 1–2 mL per min) continuously oxygenated aCSF identical to that used during cutting, and also containing 0.5–1 μM tetrodotoxin (TTX; Tocris, Bristol, UK) to isolate $GABA_A$ receptor-mediated mIPSCs.

Pyramidal cells and stratum oriens/alveus (SO/A) interneurons of the CA1 area were visually identified using a Zeiss Axioskop (Zeiss, Oberkochen, Germany) equipped with infrared differential interference contrast optics with a 40 × immersion objective coupled to an infrared camera system (Hamamatsu, Hamamatsu City, Japan). Pyramidal cells were identified due to their location in or close to stratum pyramidale, and the presence of a large apical dendrite projecting into the stratum radiatum. Putative SO/A interneurons were recognized due to their multipolar shape, and were subsequently identified by labelling with biocytin (see below). Whole-cell patch-clamp recordings were made with an Axopatch 1D amplifier (Axon Instruments, Foster City, USA). Patch pipettes for pyramidal cell recordings (final tip resistance 2–5 MΩ) were pulled from borosilicate glass capillaries (GC120F-10, Harvard Apparatus, Edenbridge, Kent, UK) and filled with (in mM): KCl, 130; Mg-ATP, 4; Na-GTP, 0.3;

Na_2-phosphocreatine, 10; HEPES, 10; final pH 7.40 adjusted with KOH. Patch pipettes for SO/A interneuron recordings (3.5–6 MΩ) contained (in mM): KCl, 130; K-gluconate, 10; Na_2ATP, 4; NaGTP, 0.3; $MgCl_2$, 2; HEPES, 10; EGTA, 0.05; and 0.5% biocytin; final pH 7.25 adjusted with KOH. Series resistance and whole-cell capacitance were monitored every 2 min during recording, and experiments were terminated if the series resistance increased by more than 30%. Series resistance was always compensated by ~80% using lag values of 6–8 μs. The drugs zolpidem (100 nM or 1 μM; Tocris), flurazepam (3 μM; Sigma-Aldrich), flumazenil (10 μM; Tocris) and 6-imino-3(4-methoxyphenyl)-1(6*H*)-pyridazinebutanoic acid hydrobromide (SR 95531, 50 μM; Tocris) were applied as described previously (Cope *et al*., 2004). Experimental data were stored on digital audio tape and subsequently digitized at 20 kHz using pClamp software via a DigiData 1200 analogue-to-digital converter (Axon Instruments). Acquired data were converted to an ASCII format and mIPSCs were detected and analysed using in-house, LabView-based software (National Instruments, Austin, TX, USA) running on a personal computer, as described previously (Jensen & Mody, 2001; Cope *et al*., 2004). The effect of a drug on the frequency of mIPSCs and the parameters of the average mIPSC were determined both in terms of absolute values and percent changes. Statistical tests are as indicated in the text. The significance for comparison in all instances was set at $P < 0.05$. Statistical tests were performed using Excel, Statistica (Statsoft, Tulsa, OK, USA) and Matlab (Natick, MA, USA). All mIPSC data are expressed as mean ± SD.

Visualization of recorded interneurons

Following the recording of putative interneurons, slices were sandwiched between two Millipore filters to avoid deformation and fixed for ~4 h in a solution containing 4% paraformaldehyde, 0.05% glutaraldehyde and 15% (v/v) saturated picric acid in 0.1 M PB (pH 7.4). Slices were then washed several times in PB, embedded in gelatine and resectioned at 60 μm thickness. Sections were incubated for at least 6 h in avidin-biotinylated HRP (Vector Laboratories) diluted 1 : 100 in Tris-buffered saline (TBS) with 0.1% Triton X-100. 3,3′-Diaminobenzidine (0.05%) was used as chromogen and 0.01% H_2O_2 as substrate in the peroxidase reaction, carried out in 0.05 M Tris buffer. Sections were then dehydrated and permanently mounted on slides. Recorded interneurons were analysed using a light microscope and the cell type identified based on axonal and dendritic patterns.

In vitro *extracellular field recordings*

Horizontal hippocampal slices were prepared as described above from γ2F77/F77 and γ2I77/I77 mice (P20.6 ± 3). However, the thickness of the slices was increased to 450 μm to preserve the cellular network better, 50 μM indomethacin was added to the ice-cold aCSF during the slicing procedure (Pakhotin *et al*., 1997), and 0 Ca^{2+} and 6 mM Mg^{2+} instead of kynurenic acid was used in the cutting aCSF. Slices were maintained at room temperature in a submerged storage chamber for at least 1 h before being transferred to a Haas-type interface chamber and maintained at 35 ± 1.5 °C at the interface between warm, humidified carbogen gas (95% O_2: 5% CO_2) and aCSF with the same composition as that used for the whole-cell patch-clamp experiments, but without kynurenic acid. The flow rate range was 0.2–0.3 mL/min. Extracellular field recordings were obtained from stratum pyramidale of the CA3 area with patch pipettes filled with oxygenated aCSF. Recordings were performed using an Axopatch-1D amplifier with pClamp acquisition software (Axon Instruments). Offset potentials were eliminated on line and/or off line. The signals were filtered at 2 kHz and digitized at 5 kHz. Bath application of carbachol (20 μM) elicited extracellular field activity that stabilized after about 1–3 h, and consisted of oscillations in the beta–gamma frequency range. Experiments were continued when the oscillations were stable and the power spectra reached >25 μV^2. Zolpidem (10 μM) or flurazepam (10–20 μM) were bath applied for up to 40 min, bicuculline (60 μM; Sigma-Aldrich) and TTX (10 μM) for up to 30 min. Power spectra were obtained from 60-s recording periods using a Fast Fourier transform algorithm contained in Spike2 software (CED, Cambridge, UK). To quantify the data, power spectra of the last 60 s before the end of both the control or drug application period were usually obtained and subsequently analysed. All drugs were diluted directly from frozen stock solutions in the superfusion medium. The diffusion of the drugs in slices kept at the interface chamber was slower than that obtained in submerged slices, and therefore the drugs were applied at concentrations several fold higher than those used in the whole-cell patch-clamp experiments. The Δpower is an absolute value calculated as the difference between the power spectrum measured during the application of zolpidem or flurazepam together with carbachol minus that measured in carbachol alone. The Δfrequency is an absolute value calculated as the difference between the frequency with the highest power measured in zolpidem or flurazepam together with carbachol minus that measured in carbachol alone. Data are presented as mean ± SE.

Results

Distribution and expression of $GABA_A$ receptor subunits in hippocampus of γ2F77/F77 and γ2I77/I77 mice

No detectable differences in the distribution or the intensity of the immunoreactivity for the $GABA_A$ receptor γ2 subunit were observed between γ2I77/I77 or γ2F77/F77 mice (Fig. 1). In order to test if the γ2I77 point mutation produced changes in the expression of other $GABA_A$ receptor subunits, we have investigated the distribution of immunoreactivity for the α1, α2, α5 and β3 subunits, which are prominently expressed in the hippocampus (Persohn *et al*., 1992; Wisden *et al*., 1992; Fritschy & Mohler, 1995; Sperk *et al*., 1997). No changes were detected in either the pattern or intensity of immunoreactivity (Fig. 1). Immunolabelling for the $GABA_A$ receptor subunits in mice, either carrying the point mutation or in control littermates, was similar to that described previously in the rat (Zimprich *et al*., 1991; Gutierrez *et al*., 1994; Fritschy & Mohler, 1995; Sperk *et al*., 1997; Pirker *et al*., 2000) and mouse (Fritschy *et al*., 1998; Baer *et al*., 2000; Bouilleret *et al*., 2000; Crestani *et al*., 2002; Schweizer *et al*., 2003).

To examine any differences in $GABA_A$ receptor expression quantitatively, we performed immunoblots on receptor subunits isolated from the hippocampus. Because immunoblot analysis measures receptor assembly intermediates as well as assembled receptors, we determined the number of assembled receptors in the hippocampus by undertaking Scatchard analysis of [^{3}H]flunitrazepam and [^{3}H]muscimol ligand binding on hippocampal membranes, and quantitative [^{3}H]muscimol and [^{35}S]TBPS autoradiography. The binding of [^{3}H]flunitrazepam is an index of αβγ2-type receptors. The binding of [^{3}H]muscimol together with the quantitative [^{3}H]muscimol autoradiography labelling are an indication of αβδ-type receptors, given that high-affinity [^{3}H]muscimol labelling is lost in δ-subunit-deficient mice (Korpi *et al*., 2002a, b). The autoradiography of [^{35}S]TBPS is an index of general $GABA_A$ receptors. Immunoblot analysis of seven $GABA_A$ receptor subunits in the hippocampus of γ2I77/I77 and

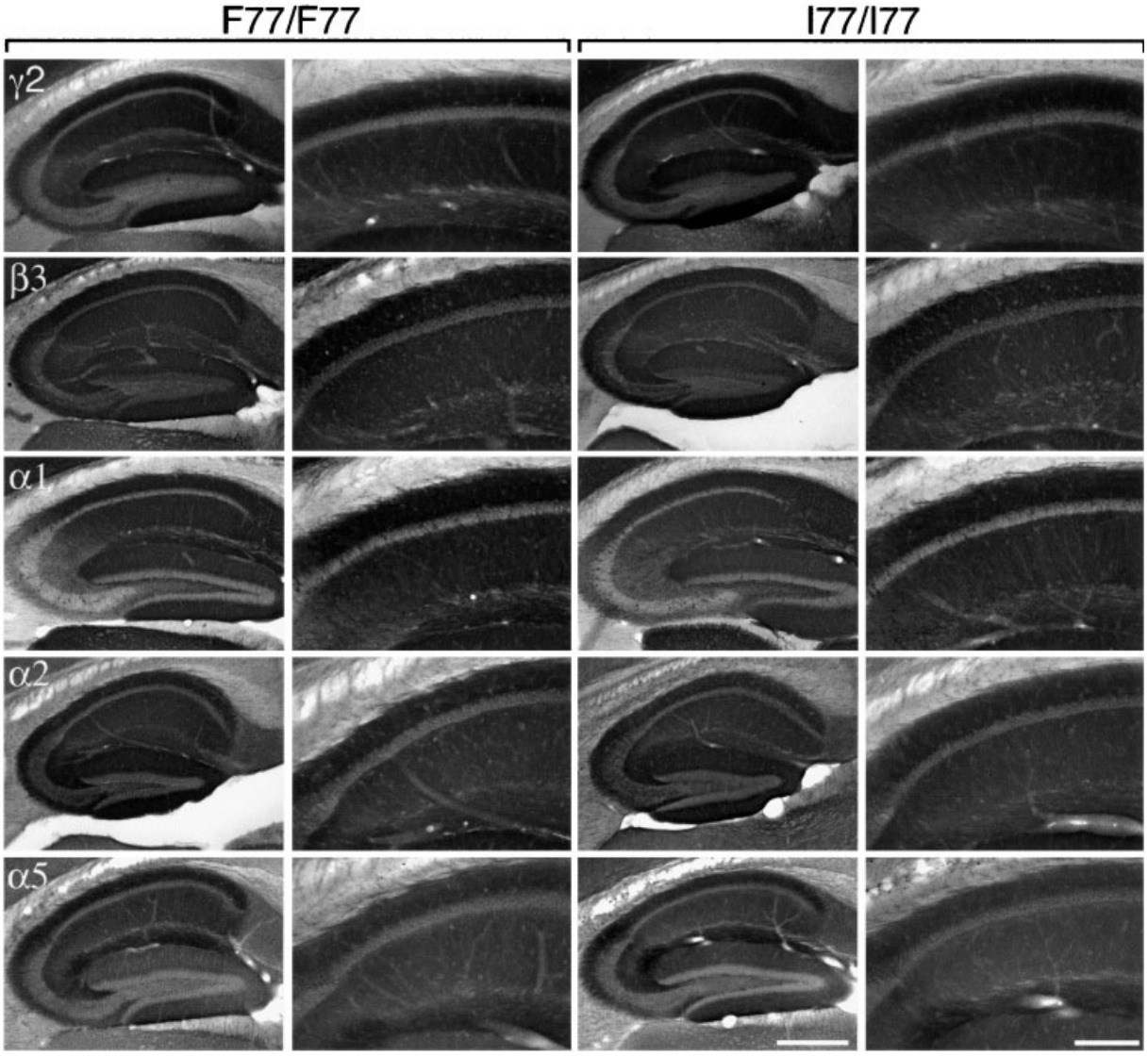

FIG. 1. Comparison of the distribution of $GABA_A$ receptor subunits in the hippocampus of γ2I77/I77 and γ2F77/F77 mice. No apparent differences were detected in the distribution or the intensity of immunolabelling for any of the subunits tested. A coronal section of the hippocampal formation and an enlarged view of the CA1 area immunolabelled for each receptor subunit are shown. Scale bars: for the entire hippocampus, 500 μm; for CA1, 200 μm.

γ2F77/F77 mice indicated a 24–36% increase ($P < 0.05$, Student's unpaired *t*-test) in the expression of the α1, α4, γ2 and δ subunits, but not the α2, β2 or β3 subunits (Table 1). Scatchard analysis of [^{3}H]flunitrazepam (1–20 nM) binding data in hippocampal membranes of γ2F77/F77 and γ2I77/I77 mice showed that the total number of [^{3}H]flunitrazepam binding sites was not significantly different between the two genotypes (γ2F77/F77: 3.1 ± 0.3 pmol/mg; γ2I77/I77: 3.9 ± 0.3 pmol/mg; $P > 0.05$, Student's unpaired *t*-test), although the affinity of [^{3}H]flunitrazepam binding was significantly lower in γ2I77/I77 compared with γ2F77/F77 mice (4.6 ± 0.7 and 2.0 ± 0.4 nM, respectively, $P < 0.05$), in accordance with previous studies (Buhr *et al*., 1997; Wingrove *et al*., 1997). In addition, we attempted to measure the total number of $GABA_A$ receptors in the hippocampus by calculating the solubilization efficacy and number of [^{3}H]muscimol (40 nM) binding sites in hippocampal $GABA_A$ receptor extracts following ultracentrifugation of hippocampal homogenates at

TABLE 1. Western blot analysis of hippocampal $GABA_A$ receptor subunit expression

	Percentage of $GABA_A$ receptors			
Subunit	γ2F77/F77	*n*	γ2I77/I77	*n*
α1	100.0 ± 4.4	6	124.1 ± 10.0*	6
α2	100.0 ± 3.2	6	97.8 ± 4.1	6
α4	100.0 ± 4.8	6	129.2 ± 4.2*	6
β2	100.0 ± 4.3	6	109.0 ± 8.6	6
β3	100.0 ± 3.6	6	103.3 ± 1.5	6
γ2	100.0 ± 3.9	6	128.4 ± 10.4*	6
δ	100.0 ± 7.7	6	135.7 ± 7.4*	6

Data are presented as mean ± SEM. *n* = number of individual animals tested. Student's unpaired *t*-test was used for comparisons between genotypes (*$P < 0.05$).

150,000 ***g***. The solubilization efficacy of combined supernatant extract and redissolved pellet was not significantly different between the two genotypes (γ2F77/F77: 83.4%; γ2I77/I77: 84.3%). There was also no significant difference in the total number of [^{3}H]muscimol binding sites between γ2F77/F77 (2.1 ± 0.4 pmol/mg) and γ2I77/I77 (3.2 ± 0.8 pmol/mg) mice ($n = 5$).

The binding of [^{3}H]muscimol to the hippocampus in coronal brain sections was similar between the γ2F77/F77 and γ2I77/I77 mice (11.5 ± 2.5 vs. 12.4 ± 1.4 nCi/mg, respectively, $n = 5$ each; $P > 0.05$, Student's unpaired *t*-test, data not shown), as was the binding of [^{35}S]TBPS (108 ± 10 vs. 101 ± 11 nCi/g, respectively, $n = 5$ each; $P > 0.05$, Student's unpaired *t*-test, data not shown). The affinity of [^{3}H]Ro 15-4513 was so low that γ2I77/I77 brain sections had only background binding levels and therefore this ligand could not be used for quantification of γ2 subunit-containing receptors.

Effects of zolpidem on mIPSCs recorded from CA1 pyramidal cells are attenuated in γ2I77/I77 mice

Results were obtained from 43 pyramidal cells from C57BL/6J mice, 46 pyramidal cells from γ2I77/I77 mice and 41 pyramidal cells from γ2F77/F77 mice. The properties of control pyramidal cell mIPSCs, i.e. mIPSCs prior to any drug application, are presented in Table 2. The peak amplitude of the average mIPSCs and the frequency of mIPSCs was significantly smaller in C57BL/6J mice compared with both γ2I77/I77 and γ2F77/F77 mice ($P < 0.05$, ANOVA with *post-hoc* Tukey HSD). There were no significant differences in control mIPSC parameters between γ2I77/I77 and γ2F77/F77 mice. The average age of the pyramidal cells recorded from C57BL/6J mice was significantly smaller than those of both γ2I77/I77 and γ2F77F77 mice (21.61 ± 0.42 days compared with 27.91 ± 0.89 and 28.32 ± 1.39 days, respectively, both $P < 0.05$). However, there was no correlation of age and peak amplitude or frequency among genotypes (Pearson's $r = -0.35$ and +0.25, respectively). The $GABA_A$ receptor antagonist SR 95531 (50 μM) completely blocked mIPSCs in γ2I77/I77 ($n = 3$ cells) and γ2F77/F77 ($n = 4$ cells) mice (data not shown), confirming they were mediated by $GABA_A$ receptors.

Bath application of the BZ site agonist zolpidem (1 μM) to a subset of pyramidal cells of C57BL/6J ($n = 22$) and γ2F77/F77 ($n = 17$) mice caused a significant increase in peak amplitude, weighted decay time constant and 10–90% rise time of the average mIPSCs, and a significant increase in the frequency of mIPSCs ($P < 0.05$, Student's paired *t*-test; Fig. 2A and B). These effects were due to the action of zolpidem because significant changes in mIPSC parameters were not observed following sham experiments in C57BL/6J mice ($n = 10$, data not shown). In pyramidal cells of γ2I77/I77 mice, 1 μM zolpidem also caused a significant, albeit much smaller, increase in the weighted decay time constant of the average mIPSCs ($n = 22$, Fig. 2A and B), but in contrast to the other two genotypes there were no significant changes in 10–90% rise time or the frequency of the mIPSCs. In addition, we observed a significant decrease in the peak amplitude of the average mIPSCs induced by 1 μM zolpidem in pyramidal cells of γ2I77/I77 mice. We calculated the percent change in each parameter elicited by 1 μM zolpidem and compared these changes among the three mouse genotypes. The increases in peak amplitude, weighted decay time constant and 10–90% rise time in the C57BL/6J and γ2F77/F77 were always significantly larger than in the γ2I77/I77 mice ($P < 0.05$, Kruskal–Wallis test with *post-hoc* Dunn; Fig. 2C). The increase in the frequency of mIPSCs was greater in C57BL/6J compared with γ2I77/I77 mice, but not in γ2F77/F77 compared with γ2I77/I77 mice. Percent changes in each mIPSC parameter were never significantly different between C57BL/6J and γ2F77/F77 mice.

Although zolpidem shows some selectivity for α1 subunit-containing receptors, at a concentration of 1 μM it may also act on α2 and/or α3 subunit-containing receptors (Pritchett & Seeburg, 1990). The α2 subunit is particularly strongly expressed in CA1 pyramidal cells, at least in the rat (Persohn *et al*., 1992; Wisden *et al*., 1992; Fritschy & Mohler, 1995; Sperk *et al*., 1997). We therefore further tested the effects of zolpidem at a concentration (100 nM) that should preferentially affect only α1 subunit-containing receptors. In C57BL/6J ($n = 11$ cells) and γ2F77/F77 ($n = 13$ cells) mice, 100 nM zolpidem caused a significant increase in the peak amplitude and weighted decay time constant of the average mIPSCs, a significant increase in 10–90% rise time in γ2F77/F77, but not C57BL/6J, mice, and a significant increase in the frequency of mIPSCs in C57BL/6J, but not γ2F77/F77, mice ($P < 0.05$, Student's paired *t*-test; Table 3). In γ2I77/I77 mice ($n = 14$ cells), 100 nM zolpidem caused a significant increase only in the weighted decay time constant. Comparison of the percent changes showed that the increase in peak amplitude and weighted decay time constant, but not those in 10–90% rise time or frequency, were significantly larger in both C57BL/6J and γ2F77/F77 mice compared with γ2I77/I77 mice ($P < 0.05$, Kruskal–Wallis test with *post-hoc* Dunn). Percent changes between γ2F77/F77 and C57BL/6J mice were never significantly different.

In order to test the specificity of zolpidem for the BZ binding site, the BZ antagonist flumazenil (10 μM) was applied in the continuing presence of zolpidem (1 μM) in a subset of pyramidal cells. In C57BL/6J ($n = 10$ cells) and γ2F77/F77 ($n = 8$ cells) mice, flumazenil completely reversed the changes in peak amplitude, weighted decay time constant, 10–90% rise time and frequency caused by zolpidem, so that values following flumazenil application were not statistically different from control values ($P > 0.05$, ANOVA, data not shown). In γ2I77/I77 ($n = 10$ cells) mice, values under the three experimental conditions (control, zolpidem, zolpidem + flumazenil) were not significantly different from each other (data not shown). However, there was a small but significant decrease in the peak amplitude and increase in the weighted decay time constant ($P < 0.05$) when the values observed in the control and in the presence of zolpidem in these cells were compared with Student's paired *t*-test, consistent with the statistical results obtained for similar data shown in Fig. 2.

To test the specificity of the γ2F77I point mutation for zolpidem, we compared the effects of the BZ agonist flurazepam (3 μM) on pyramidal cell mIPSCs of γ2I77/I77 ($n = 7$ cells) and γ2F77/F77 ($n = 7$ cells) mice. Application of flurazepam significantly increased

TABLE 2. Comparison of CA1 pyramidal cell control mIPSCs

	Mouse genotype		
mIPSC	C57BL/6J ($n = 43$)	γ2I77/I77 ($n = 46$)	γ2F77/F77 ($n = 41$)
Peak amplitude (pA)	−41.7 ± 8.2†,***	−48.0 ± 11.0	−50.3 ± 11.2
Weighted decay time constant (ms)	5.0 ± 0.6	4.8 ± 0.5	4.8 ± 0.6
10–90% rise time (μs)	320.5 ± 30.5	316.3 ± 29.8	316.6 ± 29.9
Frequency (Hz)	9.3 ± 3.1†††,***	14.1 ± 4.6	13.4 ± 5.3

Data are expressed as mean ± SD. n = number of recorded cells. Two way ANOVA with *post-hoc* Tukey HSD was used for comparisons between genotypes ($^{\dagger}P < 0.05$, C57BL/6J vs. γ2I77/I77; $^{\dagger\dagger\dagger}P < 0.001$, C57BL/6J vs. γ2I77/I77; ***$P < 0.001$, C57BL/6J vs. γ2F77/F77).

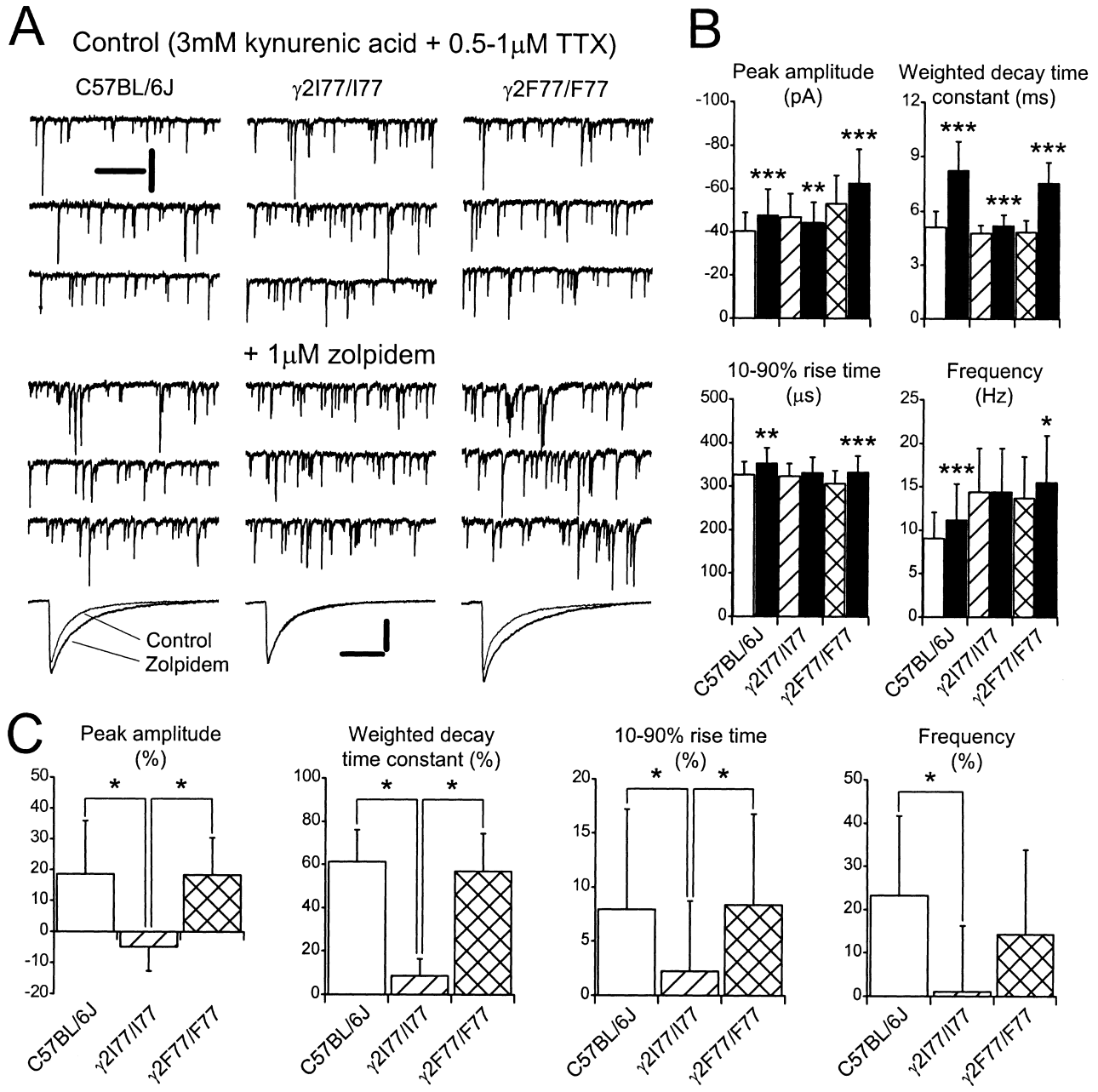

FIG. 2. Actions of zolpidem on mIPSCs recorded from pyramidal cells of the three mouse genotypes. (A) Consecutive traces of mIPSCs recorded from a CA1 pyramidal cell of a P22 C57BL/6J mouse (left), P28 γ2I77/I77 mouse (middle) and P32 γ2F77/F77 mouse (right), prior to (upper panels) and following (lower panels) the application of 1 μM zolpidem. Control mIPSCs for this figure and Figs 4 and 5 were recorded in the presence of 3 mM kynurenic acid and 0.5–1 μM tetrodotoxin (TTX). Below each column are the average mIPSCs for the cells shown before (thin line) and after (thick line) zolpidem application. For the cells from the C57BL/6J and γ2F77/F77 mice, zolpidem caused a large increase in both the peak amplitude and decay of the mIPSC, but for the cell from the γ2I77/I77 mouse the effects of zolpidem are greatly reduced. (B) Graphs showing the effects of 1 μM zolpidem (black columns) on pyramidal cell mIPSC parameters for each mouse genotype (C57BL/6J, open columns, $n = 22$ cells; γ2I77/I77, diagonally lined columns, $n = 22$ cells; γ2F77/F77, hatched columns, $n = 17$). Significant effects (Student's paired *t*-test) of zolpidem are as indicated (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). (C) Graphs comparing the percent change in mIPSC parameters between mouse genotypes (same labelling as in B). Significant differences (Kruskal–Wallis test with *post-hoc* Dunn) between genotypes are as indicated (*$P < 0.05$). Calibration bars (A): mIPSC traces, 500 ms and 100 pA; average mIPSCs, 10 ms and 20 pA.

the weighted decay time constant of the average mIPSCs in both genotypes, and the 10–90% rise time only in γ2F77/F77 mice ($P < 0.05$, Student's paired *t*-test, Fig. 3A and B). The peak amplitude of the average mIPSCs and the frequency of mIPSCs were not significantly altered. Comparison of the percent change in each mIPSC parameter between genotypes showed that only the increase in 10–90% rise time was significantly different between genotypes ($P < 0.05$, Mann–Whitney *U*-test; Fig. 3C).

Effects of zolpidem on mIPSCs of stratum oriens/alveus interneurons in γ2I77/I77 mice are also attenuated

Because hippocampal interneurons, in conjunction with pyramidal cells, are involved in the generation of behaviourally relevant network oscillations (Csicsvari *et al.*, 1999; Klausberger *et al.*, 2003; Whittington & Traub, 2003), we tested the effects of the γ2F77I point mutation on mIPSCs recorded from interneurons. We

TABLE 3. Effects of 100 nM zolpidem on CA1 pyramidal cell mIPSCs

	Mouse genotype		
mIPSC	C57BL/6J ($n = 11$)	γ2I77/I77 ($n = 14$)	γ2F77/F77 ($n = 13$)
Peak amplitude (pA)			
Control	−41.6 ± 5.5	−46.7 ± 6.1	−49.5 ± 10.0
+ 100 nM zolpidem	−46.4 ± 7.1**	−45.9 ± 8.0	−54.3 ± 9.1**
Change (%)	11.6 ± 9.2	−1.9 ± 10.7	10.8 ± 11.6
Weighted decay time constant (ms)			
Control	4.9 ± 0.4	4.7 ± 0.4	4.6 ± 0.3
+ 100 nM zolpidem	7.0 ± 0.8***	5.2 ± 0.5***	6.6 ± 1.0***
Change (%)	42.0 ± 11.7	12.5 ± 9.3	41.0 ± 17.1
10–90% rise time (μs)			
Control	306.4 ± 37.8	312.1 ± 32.8	313.8 ± 21.4
+ 100 nM zolpidem	313.6 ± 32.9	320.0 ± 28.8	332.3 ± 32.2**
Change (%)	2.8 ± 7.2	2.7 ± 4.7	5.9 ± 6.8
Frequency (Hz)			
Control	10.1 ± 4.1	12.8 ± 3.3	12.7 ± 6.4
+ 100 nM zolpidem	10.9 ± 4.9*	12.4 ± 3.8	12.7 ± 6.1
Change (%)	6.1 ± 9.3	−3.8 ± 10.9	1.8 ± 14.5

Data are expressed as mean ± SD. n = number of recorded cells. Student's paired t-test was used for comparison between pre- and post-zolpidem values within genotypes (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

recorded mIPSCs from 18 putative SO/A interneurons from C57BL/6J mice, 11 from γ2I77/I77 mice and 11 from γ2F77/F77 mice. Putative interneurons were filled with biocytin and identified by microscopic analysis of their axons (data not shown). Of the 18 SO/A interneurons from C57BL/6J mice, 10 were basket cells, two were bistratified cells, four were oriens lacunosum/moleculare (O-LM) cells and two could not be identified, but were not pyramidal cells. The 11 SO/A interneurons from γ2I77/I77 mice comprised one basket cell, four O-LM cells, three bistratified cells and three that could not be identified, but were not pyramidal cells. The 11 SO/A interneurons from γ2F77/F77 mice comprised four O-LM cells, one bistratified cell and six that could not be identified, but were not pyramidal cells. Because we did not record a sufficient number of any cell type across all genotypes, and given that the effects of zolpidem within genotypes were similar irrespective of cell type, all data from interneurons within a genotype were pooled.

The properties of control mIPSCs of SO/A interneurons prior to drug application for each genotype are shown in Table 4. There were no apparent differences in mIPSC properties between genotypes, except that the weighted decay time constant was significantly faster in γ2I77/I77 compared with γ2F77/F77 mice ($P < 0.05$, ANOVA with *post-hoc* Tukey HSD). Application of 1 μM zolpidem caused a significant increase in the peak amplitude, weighted decay time

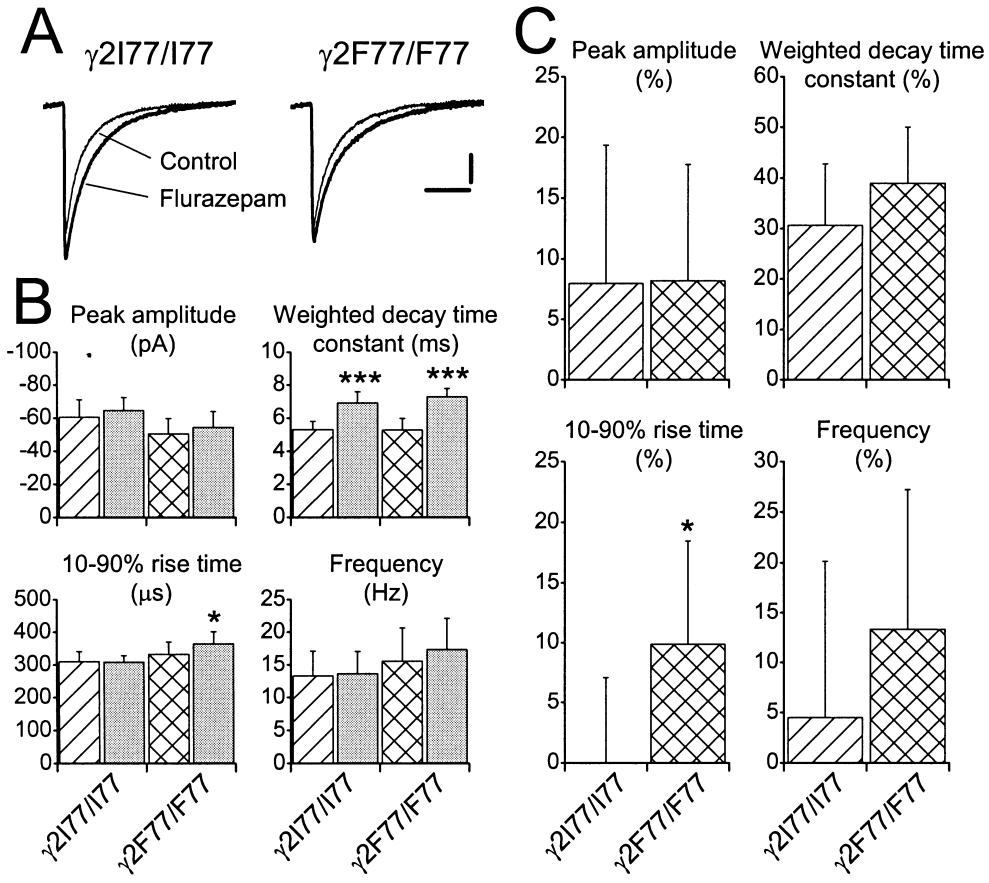

FIG. 3. Effects of flurazepam on mIPSCs recorded from CA1 pyramidal cells of γ2I77/I77 and γ2F77/F77 mice. (A) Average mIPSCs from a pyramidal cell of a P32 γ2I77/I77 mouse (left) and a P38 γ2F77/F77 mouse, prior to (thin line) and subsequent to (thick line) application of 3 μM flurazepam. Flurazepam increases peak amplitude and decay to a similar extent in both cells. (B) Graphs showing the effects of flurazepam (grey columns) on pyramidal cell mIPSC parameters of γ2I77/I77 (diagonally lined columns, $n = 7$ cells) and γ2F77/F77 (hatched columns, $n = 7$ cells) mice. Significant effects (Student's paired t-test) of flurazepam are as indicated (*$P < 0.05$; ***$P < 0.001$). (C) Comparison of the percent change in mIPSC parameters between mouse genotypes (same labelling as in B). Significant differences (Mann–Whitney U-test) are as indicated (*$P < 0.05$). Calibration bars (A),10 ms and 10 pA.

TABLE 4. Comparison of SO/A interneuron control mIPSCs

mIPSC	Mouse genotype		
	C57BL/6J ($n = 18$)	γ2I77/I77 ($n = 11$)	γ2F77/F77 ($n = 11$)
Peak amplitude (pA)	−35.1 ± 11.1	−39.6 ± 16.7	−33.6 ± 3.2
Weighted decay time constant (ms)	4.6 ± 1.8	3.6 ± 1.0*	5.6 ± 2.3
10–90% rise time (μs)	380.7 ± 55.3	360.4 ± 46.2	389.0 ± 49.6
Frequency (Hz)	4.2 ± 3.5	7.4 ± 5.3	3.8 ± 3.7

Data are expressed as mean ± SD. n = number of recorded cells. Two way ANOVA with *post-hoc* Tukey HSD was used for comparisons between genotypes (*$P < 0.05$, γ2I77/I77 vs. γ2F77/F77).

constant, 10–90% rise time of the average mIPSCs and the frequency of mIPSCs in both C57BL/6J and γ2F77/F77 mice ($P < 0.05$, Student's paired *t*-test; Fig. 4A and B) as shown previously in the rat (Patenaude *et al.*, 2001). However, zolpidem increased only the weighted decay time constant and the 10–90% rise time, but not the peak amplitude or the frequency, of mIPSCs of γ2I77/I77 mice. The increase in weighted decay time constant caused by zolpidem was significantly smaller in γ2I77/I77 compared with both γ2F77/F77 and C57BL/6J mice ($P < 0.05$, Kruskal–Wallis test with *post-hoc* Dunn; Fig. 4C). In addition, the peak amplitude and frequency were always greater in C57BL/6J compared with γ2I77/I77 mice, but not in γ2F77/F77 compared with γ2I77/I77 mice. No difference was found in 10–90% rise time between genotypes, and changes in mIPSC parameters were never different between C57BL/6J and γ2F77/F77 mice.

Actions of zolpidem on carbachol-induced oscillations in the CA3 area of γ2I77/I77 mice are attenuated

Oscillatory activity in the beta/gamma frequency range is critically dependent on the rhythmic synchronous output of populations of interneurons (Whittington *et al.*, 1995; Fisahn *et al.*, 1998). Blockers of $GABA_A$ receptors abolish this activity, and $GABA_A$ modulators, such as barbiturates, markedly reduce the frequency of the oscillations (Fisahn *et al.*, 1998). Therefore, we tested the action of zolpidem on rhythmic activity induced by the cholinergic agonist carbachol in the CA3 subfield, where these oscillations are more prominent than in the CA1 area. Bath application of carbachol (20 μM) caused rhythmic field potential oscillations (Fig. 5A), which progressively stabilized with time and were abolished either by the $GABA_A$ receptor antagonist bicuculline (60 μM) or by TTX (10 μM) in slices from both genotypes (Fig. 5A, $n = 3$). At steady state, the mean frequency of the oscillation was 21.72 ± 1.16 Hz in slices from γ2F77/F77 mice, and 24 ± 1.42 Hz in those from γ2I77/I77 mice. The mean

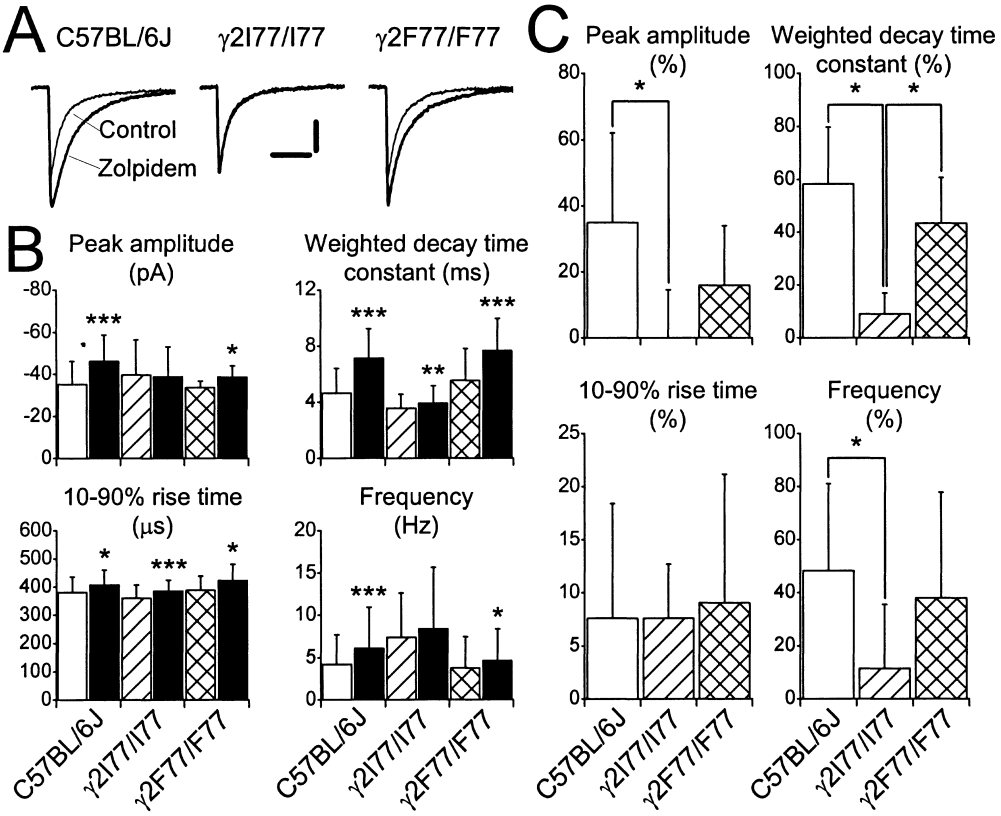

FIG. 4. Zolpidem potentiation of mIPSCs is reduced in SO/A interneurons of γ2I77/I77 mice. (A) Average mIPSCs prior to (thin line) and following (thick line) 1 μM zolpidem application to a SO/A interneuron of a P28 C57BL/6J mouse (left), a P20 γ2I77/I77 mouse (middle) and a P22 γ2F77/F77 mouse (right). Zolpidem increases peak amplitude and decay in the cells of the C57BL/6J and γ2F77/F77 mice, but not in the cell of the γ2I77/I77 mouse. (B) Graphs showing the effects of zolpidem (black columns) on SO/A interneuron mIPSC parameters for C57BL/6J mice (open columns, $n = 18$ cells), γ2I77/I77 (diagonally lined columns, $n = 11$ cells) and γ2F77/F77 mice (hatched columns, $n = 11$ cells). Significant differences (Student's paired *t*-test) are as indicated (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). (C) Comparison of the percent change in mIPSC parameters between genotypes (same labelling as in B). Significant differences (Kruskal–Wallis test with *post-hoc* Dunn) are as indicated (*$P < 0.05$). Calibration bars (A), 10 ms and 10 pA.

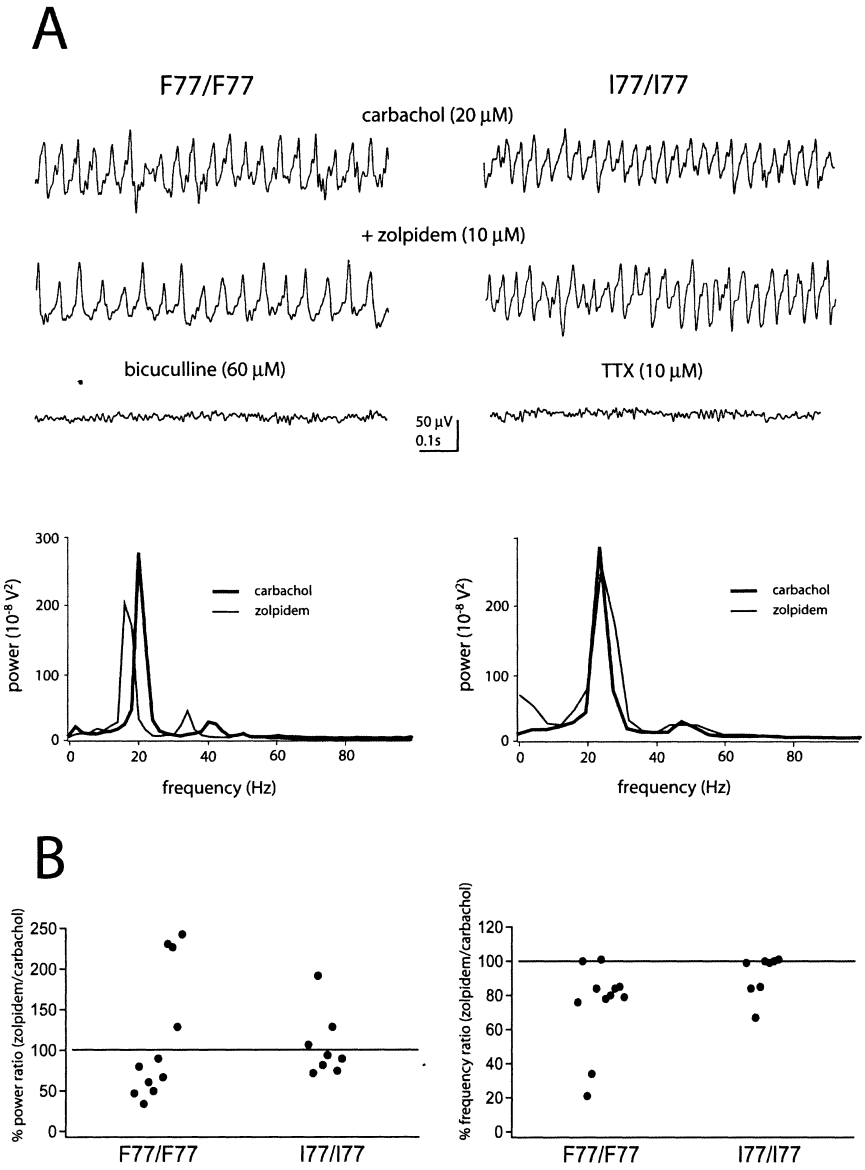

FIG. 5. Zolpidem affects carbachol-induced oscillations in γ2F77/F77, but not γ2I77/I77, mice. (A) Traces of extracellular field potential recordings from stratum pyramidale of the CA3 area. Bath application of carbachol elicits oscillations in the beta frequency range. Zolpidem reduces the amplitude and the frequency of the oscillations in the slice from a γ2F77/F77, but not in the slice from a γ2I77/I77, mouse. Bicuculline or tetrodotoxin (TTX) abolished the rhythmic activity. Lower panels show power spectra of the recorded traces above. (B) Summary of the effect of zolpidem in individual experiments (dots) expressed as ratio of power (left graph) and frequency (right graph) from slices of γ2F77/F77 ($n = 11$) and γ2I77/I77 ($n = 8$) mice. Zolpidem increases or decreases the power, and reduces the frequency of the oscillations. These effects are diminished in γ2I77/I77 compared with γ2F77/F77 mice.

power was 131 ± 24 μV^2 (γ2F77/F77, $n = 14$) and 130 ± 30 μV^2 (γ2I77/I77, $n = 10$). No significant differences in mean frequency or power of the oscillations were observed between the two genotypes ($P > 0.05$, Mann–Whitney U-test). Zolpidem (10 μM) was applied to slices showing stable oscillations in the presence of carbachol (Fig. 5A and B). In slices taken from γ2F77/F77 mice, zolpidem changed the mean power of the oscillations (mean Δpower = 64.5 ± 17.3 μV^2, $n = 11$). In particular, zolpidem reduced the power in seven slices to 61.2 ± 7.3% of control, whereas it enhanced the power in a further four slices to 207.4 ± 26.4% of control. In slices from γ2I77/I77 mice, zolpidem did not change the power of oscillations in seven slices (92.7 ± 7.5% of control), and increased it by 92% in one slice. A significant difference in the power of the oscillations before and during zolpidem (Δpower) was observed between the two genotypes ($P < 0.05$, Mann–Whitney U-test). Furthermore, zolpidem reduced the frequency of the oscillations in nine out of 11 slices to a mean frequency of 74.6 ± 7.5% of control ($n = 11$; $P < 0.05$, Wilcoxon Signed Ranks or Student's paired t-test) from γ2F77/F77 mice. In γ2I77/I77 mice no significant effect was observed; only three out of eight slices were affected (91.8 ± 4.3% of control, $n = 8$; $P > 0.05$, Wilcoxon Signed Ranks or Student's paired t-test). The change in frequency of the oscillations before and during zolpidem (Δfrequency) was found to be significantly different between the two genotypes ($P < 0.05$, Mann–Whitney U-test).

The BZ agonist flurazepam (10–20 μM) was also studied to test the specificity of the γ2F77I point mutation for zolpidem. Unlike zolpidem, application of flurazepam produced similar changes in slices from γ2F77/F77 and γ2I77/I77 mice (data not shown).

Quantitatively, flurazepam reduced the power to 77.4 ± 20.7% and 53.5 ± 14% of control in γ2F77/F77 and γ2I77/I77 mice, respectively, and decreased the frequency to 89 ± 3.4% and 95 ± 11.4% of control in γ2F77/F77 and γ2I77/I77 mice, respectively ($n = 4$ each group). These results, taken together, indicate that the effects of zolpidem on carbachol-induced beta/gamma oscillations were severely reduced in the hippocampal slices from γ2I77/I77 mice.

Discussion

Effects of zolpidem in the hippocampus are markedly reduced in γ2I77/I77 mice

In the cerebellum the γ2F77I point mutation virtually abolished the action of zolpidem on mIPSCs of Purkinje cells and stellate/basket cells, and on the ability of zolpidem to sedate mice during the rotarod test. Quantitative ligand binding and immunocytochemistry experiments did not reveal alterations in cerebellar receptor levels in γ2F77I mice, although we did observe a 15% decrease in γ2 subunit levels as determined by Western blots (Cope *et al.*, 2004). In addition, the properties of control mIPSCs in the two cell types were similar between point mutant and control mice. Thus, in the cerebellum, this gene polymorphism is largely neutral. However, Purkinje cells and stellate/basket cells are known to express only a few $GABA_A$ receptor subunits (Wisden *et al.*, 1996). Here, we tested the effect of the γ2F77I point mutation on a region that expresses many $GABA_A$ receptor subunits, the hippocampus. Indeed, rat CA1 pyramidal cells express up to 14 subunits (Persohn *et al.*, 1992; Wisden *et al.*, 1992; Fritschy & Mohler, 1995; Sperk *et al.*, 1997; Wegelius *et al.*, 1998; Ogurusu *et al.*, 1999). We report the following main observations. First, there is no significant difference in the levels of functional/assembled hippocampal $GABA_A$ receptors between the point mutant and littermate control mice as determined by receptor autoradiography with [^{3}H]muscimol and [^{35}S]TBPS binding to brain sections and Scatchard analysis on hippocampal membranes or purified receptors. In addition, there is also no change in the expression of individual receptor subunits as determined by immunocytochemistry, and the values of control mIPSC parameters in pyramidal cells and interneurons were not different between point mutant and littermate control mice. Second, the actions of zolpidem are largely abolished in pyramidal cells and SO/A interneurons of γ2I77/I77 mice. Third, all actions of zolpidem are mediated via the BZ binding site, as its effects could be reversed by flumazenil. Fourth, the point mutation does not affect the actions of all the ligands for the BZ binding site, as the effects of flurazepam were similar in point mutant and wild-type mice. Fifth, zolpidem effects on carbachol-induced network oscillations in the CA3 area are strongly reduced in slices from γ2I77/I77 mice. However, quantitative immunoblot analysis did reveal a significant increase in the expression of the γ2, α1, α4 and δ subunits. Our immunoblot experiments measured not only the functional/assembled receptors but also receptor intermediates; therefore, the changes seen might be due to increased numbers of receptor intermediates rather than functional cell surface receptors. Furthermore, we observed small but statistically significant residual effects of zolpidem on mIPSCs in pyramidal cells and SO/A interneurons recorded from γ2I77/I77 mice that were not apparent in Purkinje cells, but were present in cerebellar stellate/basket cells. Because hippocampal cells express many $GABA_A$ receptor subunits, the presence of such residual effects of zolpidem could be due to actions on diverse $GABA_A$ receptors in these cells, compared with Purkinje cells.

Effects of zolpidem on mIPSCs recorded from CA1 pyramidal cells

The control values for mIPSCs for all three genotypes tested here were similar to those described previously in whole-cell patch-clamp recordings from mouse CA1 pyramidal cells (Hájos *et al.*, 2000; Goldstein *et al.*, 2002; Wisden *et al.*, 2002). The peak amplitude of the average mIPSCs and the frequency of mIPSCs in pyramidal cells were significantly different in C57BL/6J compared with both γ2F77/F77 and γ2I77/I77 mice. The age of the recorded cells was also slightly different between C57BL/6J and both γ2F77/F77 and γ2I77/I77 mice, but there was no correlation between age and either peak amplitude or frequency, suggesting that developmental differences do not contribute to this effect. In addition, differences in recording conditions, particularly temperature, can be discounted because the kinetic properties of mIPSCs were not different between the three genotypes. The most probable cause of the different amplitude and frequency is background genetic differences between the C57BL/6J and both γ2F77/F77 and γ2I77/I77 mice. The point mutant and wild-type littermate mice were generated through a stem cell line derived from 129ola mice before successful chimeras were bred with C57BL/6J mice (Cope *et al.*, 2004). Previous reports show that the actions of BZ site ligands in mice can vary greatly depending on genotype (e.g. Garrett *et al.*, 1998; Belzung *et al.*, 2000; Griebel *et al.*, 2000).

Application of 1 μM zolpidem to pyramidal cells of C57BL/6J and γ2F77/F77 mice caused a robust enhancement of the amplitude, decay, rise-time and frequency of mIPSCs. Similar effects of zolpidem have been detected previously in recordings from CA1 pyramidal cells of mice (Hájos *et al.*, 2000; Goldstein *et al.*, 2002; Wisden *et al.*, 2002). Interestingly, we observed a mean of up to 12% residual potentiating effect of both 1 μM and 100 nM zolpidem on the weighted decay time constant of mIPSCs in pyramidal cells from γ2I77/I77 mice, suggesting that γ2 subunit-containing receptors are not the only BZ sites involved in mediating the effects of zolpidem in pyramidal cells in control mice. This is in contrast to cerebellar Purkinje cells of γ2I77/I77 mice where no residual effects were observed, but similar to cerebellar basket/stellate cells, in which a small but significant increase in weighted decay time constant was also seen (Cope *et al.*, 2004). The residual potentiating effects in pyramidal cells may be mediated by other γ subunits, particularly γ1, given that receptors containing the γ3 subunit are zolpidem-insensitive (Herb *et al.*, 1992; Lüddens *et al.*, 1994; Benke *et al.*, 1996). Unlike in Purkinje cells, the γ1 subunit is known to be expressed in pyramidal cells of the rat (Persohn *et al.*, 1992; Wisden *et al.*, 1992; Sperk *et al.*, 1997). Zolpidem has a high affinity, but low efficacy, at α2β1γ1 receptors (Wafford *et al.*, 1993). However, unlike in rat, *in situ* hybridization experiments show that γ1 subunit mRNA is not detectable, γ3 is weakly expressed and γ2 is strongly expressed in adult mouse pyramidal cells (I. Aller & W. Wisden, unpublished). It is also possible that the γ2F77 residue is not required for the binding of zolpidem at the BZ binding site formed between α2 and/or α3 subunits and the γ2 subunit, so that α2βγ2 and α3βγ2 receptors would still be zolpidem-sensitive. But as the α2 subunit is particularly strongly expressed in pyramidal cells of the rat (Persohn *et al.*, 1992; Wisden *et al.*, 1992; Fritschy & Mohler, 1995; Sperk *et al.*, 1997) and mouse (Fritschy *et al.*, 1998; Baer *et al.*, 2000; Bouilleret *et al.*, 2000; Crestani *et al.*, 2002; Schweizer *et al.*, 2003), one would then have expected to see much larger effects of zolpidem still present in the γ2I77 pyramidal cells, but this was not the case. Moreover, the general behavioural insensitivity of mice to zolpidem (Cope *et al.*, 2004; Korpi *et al.*, unpublished) argues against α2 or α3βγ2I77 receptors

retaining significant zolpidem sensitivity. Further possibilities are that the cell type-dependent membrane lipid environment subtly or even strongly influences $GABA_A$ receptor allosteric properties, as dramatically demonstrated for potassium channels (Oliver *et al*., 2004); or that receptor interaction proteins that differ in their expression between cell types affect $GABA_A$ receptor function (e.g. Everitt *et al*., 2004).

Zolpidem shows partial selectivity for $GABA_A$ receptors containing the α1 subunit (Pritchett & Seeburg, 1990). However, at a concentration of 1 μM, zolpidem will also act at receptors containing α2 and α3 subunits. We tried to isolate the effects mediated solely by α1 subunit-containing receptors by using a lower dose of zolpidem, 100 nM, that is selective for α1 subunit-containing receptors. In C57BL/6J and γ2F77/F77 mice, we still observed potentiating effects of 100 nM zolpidem, suggesting that the majority of the response to 1 μM zolpidem is mediated by α1 subunit-containing receptors, although α2 and/or α3 subunit-containing receptors may also be involved. In γ2I77/I77 mice, the degree of potentiating actions of 100 nM zolpidem on the weighted decay time constant was similar to that seen following 1 μM zolpidem. This suggests that pyramidal cells contain small amounts of highly zolpidem-sensitive synaptic receptors other than α1βxγ2 receptors. However, the identification of these receptors, perhaps by performing more detailed zolpidem dose–response experiments or examination of recombinant receptors, is beyond the scope of this study.

Furthermore, we observed a small, but significant, reduction in peak amplitude by 1 μM zolpidem in pyramidal cells of γ2I77/I77 mice, suggesting that zolpidem may have a partial inverse agonist effect. Inverse agonist effects of zolpidem have been observed on recombinant γ1 receptors (Puia *et al*., 1991) as well as in neurons preferentially expressing the γ1 subunit (Nett *et al*., 1999). The fact that we saw residual effects in pyramidal cells, which express many receptor subunits, suggests that under normal conditions zolpidem has multiple actions on different receptor subtypes, but that the potentiating effects dominate.

The increase in the peak amplitude of mIPSCs of pyramidal cells from both C57BL/6J and γ2F77/F77 mice in response to 1 μM and 100 nM zolpidem suggests that under our control conditions postsynaptic $GABA_A$ receptors are not saturated by quantal presynaptic GABA release. This is in agreement with our previous data on cerebellar Purkinje cells (Cope *et al*., 2004; but see Perrais & Ropert, 1999). In contrast to zolpidem, flurazepam did not cause a significant increase in the peak amplitude of mIPSCs in pyramidal cells of either γ2I77/I77 or γ2F77/F77 mice, but this could be due to the high variability between, and the relatively small number of, cells tested. In the γ2F77/F77 mice, the effects of 3 μM flurazepam were smaller than those of 1 μM zolpidem. This is probably due to flurazepam having a lower affinity for the BZ binding site, differences between flurazepam and zolpidem in physical–chemical properties and in the ability to penetrate the synapse, and/or because we used a concentration that did not elicit a maximal response.

Effects of zolpidem on mIPSCs recorded from SO/A interneurons

The properties of control mIPSCs in SO/A interneurons in the three genotypes were similar to those previously described (Hájos *et al*., 2000; Patenaude *et al*., 2001). We did not observe significant differences in control mIPSC properties of SO/A interneurons between genotypes, except that the weighted decay time constant was significantly slower in γ2F77/F77 compared with γ2I77/I77 mice. This may reflect a difference in the cell types recorded, but this is unlikely because the most common identified cell type recorded from both γ2F77/F77 and γ2I77/I77 mice was the O-LM cell. By comparison, the majority of cells recorded from C57BL/6J mice were basket cells, but we have not observed any significant differences between C57BL/6J mice and the other genotypes. Zolpidem produced similar effects on SO/A interneurons recorded from C57BL/6J and γ2F77/F77 mice, in spite of the difference in cell types recorded. Different cell types might express different receptor subunits and therefore could be differentially affected by zolpidem. The α1 subunit is expressed at high density in a subset of parvalbumin (PV)-positive interneurons (Gao & Fritschy, 1994), while the α3 subunit is preferentially expressed in a small population of SO/A interneurons (Brünig *et al*., 2002). However, our zolpidem concentration of 1 μM does not discriminate between α1, α2 or α3 subunit-containing receptors; therefore, any indication of selective expression of individual subunits in different cell types may be masked.

Zolpidem potentiated SO/A interneuron mIPSCs robustly in both C57BL/6J and γ2F77/F77 mice. Peak amplitude was significantly increased, again suggesting that postsynaptic $GABA_A$ receptors are not saturated by presynaptic quantal GABA release under our experimental control conditions. Changes in mIPSC parameters in SO/A interneurons of C57BL/6J and γ2F77/F77 mice were similar to those in pyramidal cells, indicating the presence of similar receptor subtypes. Both pyramidal cells and some of the PV-positive interneurons abundantly express the α1 subunit (Klausberger *et al*., 2002), although pyramidal cells are also rich in synaptic α2 subunits (Nusser *et al*., 1996; Nyíri *et al*., 2001). In the γ2I77/I77 mice, the weighted decay time constant was increased by zolpidem to a similar extent in interneurons and pyramidal cells, suggesting a similarity between receptor subtypes expressed by pyramidal cells and SO/A interneurons. Thus, SO/A interneurons may contain zolpidem-sensitive receptors lacking the γ2 subunit.

Overall, our results and previous data (Patenaude *et al*., 2001) suggest that CA1 pyramidal cells and SO/A interneurons are endowed, at the whole cell level of analysis, with similar zolpidem-sensitive $GABA_A$ receptors. This does not challenge the evidence that synapses in different membrane domains of pyramidal cells, postsynaptic to specific inputs, preferentially express certain subunits. Synapses made by PV-positive basket cells are known to be enriched in α1 subunit-containing receptors (Klausberger *et al*., 2002), whereas those made by putative cholecystokinin (CCK)-positive basket cells contain a high level of α2 subunit-containing receptors (Nyíri *et al*., 2001). Synapses on the axon initial segment innervated by axo-axonic cells express receptors containing both α1 and α2 subunits (Nusser *et al*., 1996; Somogyi *et al*., 1996). Furthermore, the presence of different receptor subunits at specific synapses has been analysed pharmacologically following paired interneuron–pyramidal cell recordings (Pawelzik *et al*., 1999; Thomson *et al*., 2000). Low concentrations of zolpidem (200–400 nM) have been shown to enhance IPSPs in pyramidal cells generated by fast spiking, presumed PV-positive, basket cells to a greater extent than those generated by regular spiking, presumed CCK-positive, basket cells, and axo-axonic cells (Thomson *et al*., 2000). The zolpidem insensitivity and general pharmacological profile of bistratified cell-generated IPSPs was suggested to be due to the expression of α5 subunit-containing receptors at these synapses (Pawelzik *et al*., 1999; Thomson *et al*., 2000). Therefore, it is possible that, given the subunit preference of zolpidem, the γ2F77I point mutation reduces the effects of zolpidem at specific synapses preferentially targeted by PV-expressing basket cells, while at other synapses the point mutation makes less or no difference to our observed residual effects. It is also important to bear in mind that interneurons receive GABAergic inputs from other interneurons that specifically target interneurons, but

additionally from interneurons that also target pyramidal cells (Freund & Buzsáki, 1996; Gulyás *et al*., 1996).

Effects of zolpidem on carbachol-induced network oscillations

The cholinergic agonist, carbachol-evoked field potential oscillatory activity *in vitro* might mimic some aspects of behaviour-contingent network oscillations *in vivo* (Whittington & Traub, 2003). The oscillations are often observed close to 40 Hz frequency (within the 30–80 Hz gamma range), but lower frequencies within the beta range (12–30 Hz) have also been reported (Shimono *et al*., 2000). This activity is much more prominent in the CA3 compared with the CA1 area (Fisahn *et al*., 1998), most likely because of the presence of substantial recurrent excitation between pyramidal cells in the former (Amaral & Witter, 1989), and perhaps it is due also to differences in the muscarinic pharmacological profile between the two areas (Volpicelli & Levey, 2004). The oscillations are generated by synchronized and integrated activity of pyramidal cells and interneurons, resulting from cholinergic actions on both ligand-gated channels and synaptic conductances (McBain & Fisahn, 2001). Antagonists of $GABA_A$ receptors abolish oscillations, and barbiturates markedly decrease their frequency (Fisahn *et al*., 1998). Here we have found that BZ site ligands, such as zolpidem or flurazepam, also affected carbachol-induced oscillations in slices from γ2F77/F77 mice. This confirms the role of $GABA_A$ receptors and interneurons in the generation of these oscillations. In particular, zolpidem caused a decrease in the frequency of the oscillations. This effect correlates well with the slowing of mIPSC kinetics in both pyramidal cells and SO/A interneurons. Because rhythmic inhibitory synaptic potentials have a pivotal role in phasing the activity of pyramidal cells (Cobb *et al*., 1995), the prolongation of IPSCs alone may well explain the decrease in the frequency of oscillations. Moreover, zolpidem either decreased or increased the amplitude of the oscillations in different slices. This effect correlates with the increase in the peak amplitude of mIPSCs by zolpidem observed in both pyramidal cells and SO/A interneurons. An enhancement of the amplitude of carbachol-induced beta oscillations has been reported also after application of diazepam (Shimono *et al*., 2000). Recently, zolpidem was reported to enhance group I metabotropic glutamate receptor-induced, but to decrease the power of carbachol-induced, oscillations in the CA3 area of rat hippocampus (Palhalmi *et al*., 2004). The variable nature of the effect of zolpidem, namely increase or decrease of the amplitude of oscillations, could be due either to a heterogeneity in the network of interneurons surviving the slicing procedure in different slices and/or to a partial inverse agonist effect of zolpidem in neurons preferentially containing the γ1 subunit. In the network zolpidem may cause multiple actions on different receptor subtypes, as discussed previously.

Actions of zolpidem on the frequency and the amplitude of carbachol-induced oscillations were significantly reduced in γ2I77/I77 mice. This is in agreement with a substantial reduction of the zolpidem-mediated effects on the kinetics of mIPSCs recorded from CA1 pyramidal cells and SO/A interneurons from γ2I77/I77 mice. Importantly, this result suggests that interneurons acting on zolpidem-sensitive $GABA_A$ receptors contribute to carbachol-induced beta/gamma oscillations in the CA3 area of hippocampus.

In conclusion, we provide here pharmacological and functional evidence that the potent hypnotic zolpidem loses its efficacy in the hippocampus of mice with the $GABA_A$ receptor γ2 subunit F77I mutation, whereas the BZ flurazepam is still active. This extends our previous reports where we have analysed the effects of the mutation in several brain areas and with different BZ binding site ligands (Cope *et al*., 2004; Ogris *et al*., 2004; Leppä *et al*., 2005). In all neuronal types examined, the actions of zolpidem in potentiating $GABA_A$ receptor-mediated currents have been virtually abolished, although small effects, dependent on cell type, remain. These residual effects, however, do not influence the effect of zolpidem on whole-animal behaviour. For instance, γ2I77/I77 mice retain the ability to perform the rotarod test following zolpidem administration, whereas zolpidem remains a strong sedative/hypnotic in wild-type mice (Cope *et al*., 2004). We propose to utilize the γ2I77/I77 line so that zolpidem sensitivity can be selectively restored to specific neuronal types. Zolpidem administration to such animals, or application to brain slices derived from them, will enable us to reversibly modulate the activity of these specific cell populations, and may allow us to dissect the role(s) of these cell types in circuit function.

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Abbreviations

aCSF, artificial cerebrospinal fluid; BZ, benzodiazepine; CCK, cholecystokinin; GABA, γ-aminobutyric acid; HRP, horseradish peroxidase; IPSP, inhibitory postsynaptic potential; mIPSC, miniature inhibitory postsynaptic current; O-LM, oriens lacunosum/moleculare; PB, phosphate buffer; PBS, phosphate-buffered saline; PV, parvalbumin; SO/A, stratum oriens/alveus; TBPS, t-butylbicyclophosphorothionate; TBS, Tris-buffered saline; TTX, tetrodotoxin.

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