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1. Generation of γ2I77lox mice

γ2I77lox mice were generated as described¹, except that the targeting vector contained a loxP site located 5′ of exon 4 (Fig. S1a). The loxP oligonucleotide, incorporating a 5′ EcoRV site (5′-GATATCCATAACTTCGTATAGCATACATTATACGAAGTTAT-3′ (the EcoRV site is underlined), was ligated into the MscI site in intron 3. Linearized targeting vector was electroporated into RI embryonic stem cells. Cells were grown on feeder fibroblasts and selected in neomycin-containing medium. Correctly targeted ES cell clones were identified by Southern blot analysis (Fig. S1b). One of the positive clones was injected into C57BL/6 blastocysts. Male chimeras were crossed with deleter mice expressing enhanced-FLP recombinase (Ref 2; Jackson Laboratory) to remove the neomycin resistance cassette. The FLP transgene was bred out by backcrossing to C57BL/6 mice for 2 generations. Heterozygous mice were then intercrossed to generate homozygous γ2I77lox mice. PCR with primers flanking exon 4 and the 5′ loxP sites on genomic DNA from homozygous γ2I77lox mice, and sequencing of the products confirmed the presence of the F to I mutation at position 77 in exon 4 and the 5′ loxP site. Mice were genotyped by PCR analysis of genomic DNA from tail biopsies (Fig. S1c) using the following primer: γ2I77lox_s (5′-GTCATGCACATATCCTACAGTTG-3′) and γ2I77lox_as (5′-GGATAGTGCACTCAGCAGATAAG-3′).
2. The zolpidem-sensitive $\gamma_2 F77^{GFP}$ subunit and the generation of $L7\gamma_2 F77^{GFP}$ transgenic mice

For $\gamma_2 F77$ subunit transgene expression, we used a zolpidem-sensitive GABA$_A$ receptor $\gamma_2 L$ subunit that was N-terminally-tagged with EGFP$^3$, permitting us to distinguish this subunit from the endogenous $\gamma_2 I77$ subunit expression in the $\gamma_2 I77$lox brains. The $\gamma_2 F77^{GFP}$ construct encodes an N-terminal EGFP fusion with the wild-type mouse $\gamma_2 L$ subunit, such that the EGFP reading frame, positioned between amino acids 4 and 5 of the mature $\gamma_2$ peptide, follows the predicted signal peptide cleavage site; a 9E10 (myc) epitope tag is placed just after the EGFP$^3$; “L” denotes the long-splice version of the large cytoplasmic loop of the subunit. This $\gamma_2 F77^{GFP}$ subunit was previously demonstrated in HEK cells and for transfected hippocampal neurons in primary culture to assemble into functional GABA$_A$ receptors that were benzodiazepine (diazepam)-sensitive$^3$. The properties of this receptor did not differ significantly from untagged recombinant receptors$^3$.

Three restriction sites in the $L7\Delta$AUG plasmid$^4$ were modified: The 5′ HindIII site was changed to SalI, the BamHI site in exon 4 was changed to EcoRI, the 3′ EcoRI site was changed to NotI. The $\gamma_2 F77^{GFP}$ reading frame was cloned into the EcoRI site in exon 4 of the $L7$ cassette (Fig. S1d). The transgene construct was excised from the vector by digestion with SalI/NotI, purified after electrophoresis using GELase (Epicentre Technologies, Madison, WI, USA) and injected at a concentration of 0.3 $\mu$g/ml into the pronuclei of $B6D2/F1Crl \times C57BL/6NCrl$ mouse two-cell embryos. One $L7F77\gamma_2^{GFP}$ transgenic mouse line with strong and restricted expression of $\gamma_2 F77^{GFP}$ in cerebellar Purkinje cells was used in the subsequent breedings. Mice were crossed to $C57BL/6$ for 2 generations and then crossed with $\gamma_2 I77$lox mice. Mice were genotyped by Southern blot analysis of EcoRI-digested genomic tail DNA hybridized with a probe specific for GFP (Fig. S1e). Mice carrying the $L7Cre$ transgene were identified by Southern blot analysis of BamHI-digested genomic tail DNA hybridized with a probe specific for Cre recombinase (Fig. S1e).
3. In situ hybridization

The oligonucleotide sequences used were:

\[ \gamma_2-\text{Ex4}: 5'\text{-GTGTCTGGAATCCAGATTTCACCATATTGGCTATTCAAC-3'} \]

\[ \text{GFP: 5'\text{-ATGCGGTTCACCAGGGTGTCGCCCTCGAACTTCACCTCGCGGTT-3'}} \]

Images were generated from 2 to 4 week exposures to Biomax MR x-ray film (Eastman Kodak, Rochester, NY). To assess non-specific labeling of the sections, each labeled oligonucleotide was hybridized to brain sections with a 100-fold excess of unlabeled oligonucleotide.

4. EGFP immunocytochemistry, EGFP imaging and β-galactosidase staining

For the images in Fig. S2 and Fig. S3, adult mice were transcardially perfused with 4% paraformaldehyde in PBS, pH 7.4. Brains were removed and 60-μm-thick sagittal sections were cut using a Leica VT1000S vibratome. Free-floating sections were washed in PBS three times for 10 min, permeabilized in PBS plus 0.4% Triton X-100 for 30 min, blocked by incubation in PBS plus 4% normal goat serum (NGS), 0.2% Triton X-100 for 30 min (all at room temperature) and subsequently incubated with a rabbit polyclonal anti-EGFP (1/1000 dilution, Molecular Probes) primary antibody in a solution composed of 2% NGS, 0.1% Triton X-100 in PBS for 24 h at 4°C. Incubated slices were washed three times in PBS plus 1% NGS for 10 min at room temperature, incubated for 2 h at room temperature with a 1:800 dilution of a Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) in PBS plus 1.5% NGS, and subsequently washed twice in PBS plus 1% NGS and twice in PBS alone for 10 min at room temperature. Slices were rinsed briefly in 10 mM TRIS-HCl, mounted on slides, embedded in Mowiol, cover-slipped, and analyzed using an upright fluorescent microscope (Zeiss Axioplan 2; Zeiss) equipped with a Zeiss filter set 10 for detection of EGFP (excitation filter BP 450–490; dichroic mirror FT 510; emission filter BP 515–565) and filter set 15 for detection of Cy3 (excitation filter BP 546/12; dichroic mirror FT 580; emission filter LP 590).

For β-galactosidase staining (Fig. S2), 60 μm free-floating sections were incubated in 5-bromo-4-chloro-3-indolyl-β-galactoside (X-Gal) for 30-60 min. After X-Gal staining some sections were counterstained with neutral red (Sigma).
5. Immunocytochemistry: triple labeling

5.1 EGFP, GAD and α1 subunit labeling. Two female PC-γ2–swap mice and two wild-type control littersmates were deeply anaesthetised with a mixture of fentanyl citrate and midazolam hydrochloride dissolved in sterile water (1:1:2 ratio, respectively, i.p., 0.1 ml per 10 g). Animals were perfused transcardially with saline followed by a fixative composed of 2% paraformaldehyde, ~0.2% picric acid in 0.1 M phosphate buffer (PB, pH 7.2-7.4) for 15 min. After perfusion, the brains were removed, rinsed extensively in PB, and sectioned in the sagittal or frontal planes on a vibratome at 70 µm thickness. For triple-immunofluorescence, free-floating sections were incubated in blocking solution of 20% normal donkey serum (NDS) diluted in tris buffered saline (TBS, pH 7.4, 0.3% Triton) for 1 hour. The sections were then incubated in the following cocktail of primary antibodies overnight at 4° C: affinity purified rabbit antibody (0.5 µg/ml) to the α1 subunit (see Methods), guinea pig antiserum to EGFP (1:100, a gift from Dr Ryohei Tomioka, RIKEN, Japan) and sheep antibody to GAD (1:1000, see Methods). The following day, the sections were rinsed thoroughly in TBS and then incubated in a cocktail containing donkey anti-rabbit Cy5 (1:250, Jackson ImmunoResearch), donkey anti-sheep Cy3 (1:300, Jackson ImmunoResearch), and donkey anti-guinea pig Alexa 488 (1:1000, Invitrogen) for 2 hours at room temperature. Finally, the sections were rinsed in PB and mounted in Vectashield (Vector Laboratories, Burlingame, CA). Antibody specificity is described in the cited papers. For method specificity, sections were incubated in one primary antibody with the full set of three secondary antibodies and images were acquired at all wavelengths. No cross-reactivity was found.

5.2 Image acquisition. Twelve-bit, 1024x1024 pixel images were acquired with a Zeiss LSM 510/ Axiovert 100 M confocal microscope using a 63 × 1.4 NA oil immersion lens. Optical slices were scanned and recorded for three fluorophores with the pinhole sizes chosen to keep the slices 0.7 µm thick for all the three channels. Two subsequent optical slices, which had 50% overlap, were orthogonally projected and used as a 2D image. Output signals from 2 subsequent scan lines were averaged. A multi-track and multi-channel scanning procedure was used. Channel settings were for Alexa488, Argon laser (488 nm, Lasos LGK 7812 ML-1/LGN 7812, 25 mW), emission filter LP505; for Cy3, HeNe laser 1 (543 nm, Lasos LGK 7786 P, 1 mW), emission filter LP560; for Cy5, HeNe laser 2 (633 nm Lasos LGK 7628–1, 5 mW), emission filter LP650. For each track, channel separation was tested by systematic cross-excitation and detection between the channels.
6. Electrophysiology

6.1 Slice preparation and whole-cell patch-clamp recording. Adult male and female mice, aged between postnatal days 68 and 169 (P68-169), were anaesthetised with isoflurane and decapitated. The brains were removed and dissected in cold (0.5-4°C) oxygenated ‘slicing’ solution, containing (in mM): 85 NaCl, 2.5 KCl, 0.5 CaCl\(_2\), 4 MgCl\(_2\), 25 NaHCO\(_3\), 1.25 NaH\(_2\)PO\(_4\), 75 sucrose, 25 glucose, 0.01 D-(-)2-amino-5-phosphonopentanoic acid (D-AP5); pH 7.4, when bubbled with 95% O\(_2\) and 5% CO\(_2\). Parasagittal slices (200-250 µm) were cut from the cerebellar vermis by means of a vibrating blade microtome (HM 650V; Microm International GmbH, Walldorf, Germany). Slices were incubated at 32°C for 40 minutes and thereafter at room temperature, during which time the sucrose containing slicing solution was gradually replaced by a normal ‘external’ solution containing (in mM): 125 NaCl, 2.5 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 25 NaHCO\(_3\), 1.25 NaH\(_2\)PO\(_4\), and 25 glucose; pH 7.4, when bubbled with 95% O\(_2\) and 5% CO\(_2\).

Individual slices were transferred to a submerged recording chamber and perfused with oxygenated external solution (1.5-2.5 ml/min). Neurons were directly visualized under infrared differential interference contrast optics (Zeiss Axioscop; Zeiss, Oberkochen, Germany or Olympus BX51 WI; Olympus, London, UK). All recordings were made at near physiological temperature (34-38°C), except those from PC-Δγ2 mice and their littermates, which were made at room temperature (22-25°C). Whole-cell patch-clamp recordings were made with Axopatch-200A or 200B amplifiers (Molecular Devices Corporation, Sunnyvale, CA). For recordings from Purkinje cells, patch electrodes were pulled from thin-walled borosilicate glass tubing (1.5 mm o.d., 1.17 mm i.d.; G150TF-3; Warner Instruments Inc., Hamden, CT, USA), coated with Sylgard resin (Dow Corning 184), and fire polished to give a final pipette resistance of 2-4 MΩ. For molecular layer interneurons, electrodes were pulled from thick-walled glass (1.5 mm o.d., 0.86 mm i.d; GC-150F; Harvard Apparatus Ltd, Edenbridge, UK), coated with Sylgard, and fire-polished to 8-10 MΩ. Series resistance compensation (>60%; 6-9 µs lag) was used in all recordings. The ‘internal’ (pipette) solution contained (in mM): 140 CsCl, 4 NaCl, 0.5 CaCl\(_2\), 10 N-2-hydroxyethylpiperazine-N’-2-ethanesulphonic acid (HEPES), 5 ethyleneglycol-bis (β-aminoethylether)-N,N,N’,N’-tetraacetic acid (EGTA), 2 Mg-ATP; pH 7.3 with CsOH. In a few Purkinje cell recordings, tetraethylammonium (TEA) chloride (10 mM), N-(2,6-dimethylphenylcarbamoylmethyl)trialkylammonium bromide (QX314; 1 mM) and (±)-methoxyverapamil hydrochloride (D600; 0.5 mM) were added to the pipette solution to block voltage-gated potassium, sodium and calcium channels. No differences were noted, and data were pooled. No correction was made for liquid junction potential.
Miniature IPSCs (mIPSCs) were recorded at –70 mV in the presence of 5 µM 6 cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX), 10 µM D-AP5 and 0.5-1 µM tetrodotoxin (TTX). In all cases tested, mIPSCs recorded under such conditions were completely blocked by 10-20 µM of the GABA<sub>A</sub> receptor antagonist SR 95531 (gabazine).

6.2 Data analysis. Signals were recorded onto digital audiotape (DTR-1204; BioLogic, Claix, France; DC to 20 kHz); for analysis, replayed signals were filtered at 2 kHz (3dB, 8-pole lowpass Bessel) and digitised at 10 kHz (Digidata 1200; Axotape, Molecular Devices). Synaptic currents were detected using scaled template detection, implemented in IGOR Pro 5.0 (Wavemetrics, Lake Oswego, OR) with NeuroMatic 1.91 (http://www.neuromatic.thinkrandom.com). The detection criterion was set to include a large fraction of false positives. All detected events were subsequently assessed by eye and a minimum amplitude threshold was set at 3× the SD of an event-free epoch. In recordings from PC-Δγ2 mice, no mIPSCs were detected, even when the detection criterion was made progressively less stringent.

Measurements of mIPSC properties were made from averaged waveforms generated from at least 70 selected events (typically 100-300), aligned on their 20% rise times. Events were included if they had a monotonic rise and if their decay was not contaminated by subsequent events. The decay of averaged mIPSCs was best described by the sum of two exponential functions according to:

\[ y = A_1 \exp\left(\frac{-(x-x_0)}{\tau_1}\right) + A_2 \exp\left(\frac{-(x-x_0)}{\tau_2}\right) \]

where \(x_0\) is the decay onset, \(\tau_1\) and \(\tau_2\) are the decay time constants of the fast and slow components, and \(A_1\) and \(A_2\) are their respective amplitudes. The weighted time constant of decay (\(\tau_w\)) was calculated according to:

\[ \tau_w = \tau_1\left(\frac{A_1}{A_1+A_2}\right) + \tau_2\left(\frac{A_2}{A_1+A_2}\right) \]
All drugs for electrophysiological experiments were obtained from Tocris Bioscience (Bristol, UK), except D600 which was from Sigma (Poole, UK). Zolpidem was initially dissolved in dimethyl sulfoxide (DMSO) at a concentration of 50 mM and used at 1 µM (final DMSO concentration 0.002%). Zolpidem was applied to each slice only once, and its effect on mIPSCs determined only after equilibration for at least 2 minutes.

7. Mouse behavioural experiments

The animals were housed (1-5 per cage) in transparent polypropylene macrolon cages with standard rodent pellets (Harlan Teklad Global Diet, Bicester, UK) and tap water ad lib. Lights were on from 7 a.m. to 7 p.m.

7.1 Basic behavioural characterization. Basic physiological and behavioural characterization of mouse phenotypes was performed according to a modified version of the primary observational screen described in the SHIRPA protocol. The person who observed and recorded the behaviour was blind to the genotype of the animals. Particular emphasis was placed on motor characteristics: gait, spontaneous locomotor activity, horizontal wire test and righting reflex. No significant differences between the mouse lines γ2I77lox, PC-Δγ2, and PC-γ2–swap were observed for: body position, spontaneous activity, respiration, tremor, defecation, urination, transfer arousal, locomotor activity, palpebral closure, piloerection, gait, pelvic elevation, tail elevation, startle reflex, touch escape, positional passivity, trunk curl, limb grasping, visual placing, grip strength, pinna reflex, cornea reflex, toe pinch reflex, wire manoeuvre, provoked biting, righting reflex, contact righting reflex, negative geotaxis, body temperature, fear, irritability, aggression or vocalization. This indicates that the gene modifications did not induce any abnormal phenotypes.

7.2 Motor tests. The mice were trained during 7 days (6 trials per day) to stay on a rotating rod (diameter 4 cm, Rotamex 4/8, Columbus Instruments, Ohio, USA) for 180 s, with the rotation speed being accelerated from 5 to 30 rpm. The latency to fall from the rod in each trial was recorded and a daily average of 6 trials was calculated for each animal. The mice were also trained to walk along a 100-cm-long narrow wooden beam (0.8 cm in diameter) to the home cage on 4 consecutive days, 2 trials per day. If a mouse did not move within 10 s, it was gently pushed to induce movement. If the mouse fell from the beam, it was caught to prevent it from landing on the floor, and returned onto the beam. The latency to traverse the beam, i.e., to reach the other end of the beam, was recorded.
Zolpidem tartrate (Sanofi-Synthelabo AB, Bromma, Sweden) was crushed from a tablet and suspended in physiological saline. Flumazenil (Tocris Bioscience, Bristol, UK) was suspended in 3 % Tween 80 and brought to concentration with physiological saline. All substances were injected at 10 ml/kg body weight.

References


