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# Behavioural correlates of an altered balance between synaptic and extrasynaptic $GABA_A$ergic inhibition in a mouse model

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## Abstract

$GABA_A$ receptors mediate fast phasic inhibitory postsynaptic potentials and participate in slower tonic extrasynaptic inhibition. Thy1α6 mice with ectopic forebrain expression of $GABA_A$ receptor α6 subunits exhibit increased extrasynaptic $GABA_A$ receptor-mediated background conductance and reduced synaptic $GABA_A$ receptor currents in hippocampal CA1 neurons [W. Wisden *et al.* (2002) *Neuropharmacology* 43, 530–549]. Here we demonstrate that isolated CA1 neurons of these mice showed furosemide-sensitivity of GABA-evoked currents, confirming the functional expression of α6 subunit. In addition, receptor autoradiography of the CA1 region of Thy1α6 brain sections revealed pharmacological features that are unique for α6βγ2 and α6β receptors. The existence of atypical α6β receptors was confirmed after completely eliminating $GABA_A$ receptors containing γ1, γ2, γ3 or δ subunits using serial immunoaffinity chromatography on subunit-specific $GABA_A$ receptor antibodies. Behaviourally, the Thy1α6 mice showed normal features with slightly enhanced startle reflex and struggle-escape behaviours. However, they were more sensitive to $GABA_A$ antagonists DMCM (shorter latency to writhing clonus) and picrotoxinin (shorter latency to generalized convulsions). Tiagabine, an antiepileptic GABA-uptake inhibitor that increases brain GABA levels, delayed picrotoxinin-induced convulsions at a low dose of 3.2 mg/kg in Thy1α6 mice, but not in control mice; however, the overall effect of higher tiagabine doses on the convulsion latency remained smaller in the Thy1α6 mice. Altered balance between extrasynaptic and synaptic receptors thus affects seizure sensitivity to GABAergic convulsants. Importantly, the increased extrasynaptic inhibition, even when facilitated in the presence of tiagabine, was not able fully to counteract enhanced seizure induction by $GABA_A$ antagonists.

## Introduction

Disturbed γ-aminobutyric acid (GABA)-mediated inhibition may be involved in many neuropsychiatric disorders (Kaschka *et al*., 1995; Rice *et al*., 1996; Gibbs *et al*., 1997; Holopainen *et al*., 2001; Dhossche *et al*., 2002). Inhibition is mediated by two main receptor types: ionotropic type A [$GABA_AR$ (Hevers & Lüddens, 1998)] and metabotropic type B [$GABA_BR$ (Couve *et al*., 2000)]. Traditionally, fast inhibition is mediated by $GABA_ARs$ and slower, long-lasting inhibition by $GABA_BRs$. However, the $GABA_A$ergic inhibition can be dissected to fast and slow components, i.e. to synaptic 'point-to-point' inhibition and persistent tonic (background) inhibition, respectively (Brickley *et al*., 1996; Wall & Usowicz, 1997; Mody, 2001). Tonic inhibition mainly derives from extrasynaptic receptors. Its contribution to the total inhibition varies in different brain regions. In the cerebellar granule cells up to 90% of the inhibition is tonic (Hamann *et al*., 2002). Extrasynaptic receptors are activated by spill-over GABA from synapses (Rossi & Hamann, 1998). GABA transporters cannot eliminate all extracellular GABA (Attwell *et al*., 1993) and neurons are bathed at about 1 μM GABA (Lerma *et al*., 1986). GABA may act differently on synaptic and extrasynaptic receptors, given that peak GABA concentrations in the synaptic cleft lie somewhere from 300 μM (Perrais & Ropert, 1999) to over 3 mM (Mozrzymas *et al*., 1999), whereas most $GABA_AR$ subtypes have $EC_{50}$ values for GABA below 50 μM (Hevers & Lüddens, 1998).

Various $GABA_AR$ subtypes, many of which have a very high GABA affinity (Hevers & Lüddens, 1998), can produce extrasynaptic inhibition: α6βδ subtype in cerebellar granule cells (Brickley *et al*., 2001), α4δ in hippocampal dentate gyrus and thalamic neurons (Stell *et al*., 2003), α5β2/3γ2 in hippocampal pyramidal cells (Caraiscos *et al*., 2004) and α1βγ2 in hippocampal interneurons (Semyanov *et al*., 2003). The Thy1α6 mouse line (Wisden *et al*., 2002) expresses the normally cerebellar granule cell-restricted $GABA_AR$ α6 subunit ectopically under the control of Thy-1.2 promoter (Caroni, 1997). The α6 protein appears in forebrain regions, such as the deep layers of neocortex, subiculum and hippocampus (Wisden *et al*., 2002), the hippocampal CA1 region showing the highest transgene expression. It

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is almost solely located extrasynaptically on soma and dendrites, causing increased $GABA_A$ergic extrasynaptic inhibition of the CA1 pyramidal neurons. Moreover, the CA1 neurons of Thy1α6 mice show reduced frequency of spontaneous inhibitory postsynaptic currents and decreased amplitudes of miniature inhibitory postsynaptic currents (Wisden *et al*., 2002), indicating decreased synaptic $GABA_AR$ inhibition. In keeping with exclusive extrasynaptic α6 localization in the CA1 neurons of Thy1α6 mice by electron microscopic immunogold labelling, synaptic inhibition was unaffected by the α6-selective antagonist furosemide (Wisden *et al*., 2002). Thus, the Thy1α6 mouse line represents an animal model with innate alteration in the balance between tonic and phasic inhibition, especially in the CA1 region of the hippocampus.

The behavioural significance of extrasynaptic inhibition is still unclear. Therefore, we used Thy1α6 mice to investigate the influence of altered balance between synaptic and extrasynaptic inhibition on mouse behaviour. We also show the functional activity of ectopic α6 receptors and reveal their probable subunit combinations.

## Materials and methods

### *Animals and chemicals*

All experiments were undertaken with permission of the Western Finland Provincial Government and the Institutional Animal Use and Care committee of the University of Turku, Turku, Finland. We used male and female control C57BL/6 mice ($n = 86$ and 47, respectively; Harlan UK, Bicester, UK) and Thy1α6 mice [background: Thy1α6 transgene was inserted into strain CBA/cba × C57BL/6 egg genome and the Thy1α6 founder line was expanded in C57BL/6 background (Wisden *et al*., 2002); $n = 85$ and 61 for males and females, respectively; Harlan UK] at the age of 2–5 months (unless otherwise stated). Chemicals were purchased from Sigma (St. Louis, MO, USA) unless stated otherwise.

### In situ *hybridization*

For *in situ* hybridization (ISH) experiments, naïve male ($n = 8$) and female ($n = 13$) Thy1α6 mice were decapitated, whole brains were carefully dissected out, rinsed in ice-cold saline and frozen on dry ice. The frozen brains were wrapped in plastic, and then stored at −80 °C. Fourteen-micrometre frontal sections from were cut in a Microm HM 500 OM cryostat (Microm International GmbH, Walldorf, Germany), thaw-mounted onto poly-L-lysine-coated slides, and dried at room temperature (RT) for 1–2 h. Sections were fixed in 4% paraformaldehyde, washed in phosphate-buffered saline for 5 min, dehydrated in 70% ethanol for 5 min and stored in 95% ethanol at 4 °C until used in hybridization. ISH for detection of $GABA_A$ receptor subunit transcripts in the Thy1α6 mouse hippocampus was done using the protocol described by Wisden & Morris (1994). In detail, a 45-base-long antisense oligonucleotide probe complementary to mouse α6 subunit cDNA sequence (nucleotides 1291–1335; GenBank accession number NM_008068) was synthesized (CyberGene Ab, Huddinge, Sweden). The probe was [α-$^{33}$P] (NEN Life Science Products, Boston, MA, USA) 3′ end-labelled with terminal transferase (Promega Corporation, Madison, WI, USA). Unincorporated nucleotides were separated by ProbeQuant G50 Micro Columns (Amersham Biosciences Corp., Piscataway, NJ, USA) and labelling efficiency was determined with a scintillation counter. Hybridization buffer (100 μL; 50% formamide, 10% dextran sulphate, 4× SSC) containing diluted probe (0.06 fmol/μL) was applied to each slide, and hybridized under parafilm coverslips overnight at 42 °C. Sections were then washed in 1× SSC at RT for 10 min, in 1× SSC at 55 °C for 30 min, and finally through 3-min washing steps at RT as follows: 1× SSC, 0.1× SSC, 70% ethanol, 95% ethanol. Sections were then air-dried and exposed to Biomax MR films (Eastman Kodak, Rochester, NY, USA) with $^{14}$C standards. Specificity of the probe was confirmed with 100 × excess of unlabelled probe.

### *Ligand autoradiography*

Horizontal sections 14 μm thick from male mice brain ($n = 12$ and 13 for control and Thy1α6, respectively) were cut in a cryostat. Sections were thaw-mounted onto gelatin-coated object glasses, and stored frozen under desiccant at −20 °C. [$^3$H]Ro 15-4513 binding assay was performed to reveal the distribution of all benzodiazepine sites and the diazepam-insensitive α6βγ2 subunit-containing receptor population, as previously described (Mäkelä *et al*., 1997). This assay detects only poorly the α4βγ2 subunit-containing receptors for which a higher ligand concentration should have been used (Benke *et al*., 1997). Brain sections were preincubated in an ice-water bath for 15 min in 50 mM Tris/HCl (pH 7.4) supplemented with 120 mM NaCl. Final incubation in the preincubation buffer was performed with 10 nM [$^3$H]Ro 15-4513 (PerkinElmer Life Sciences Inc., Boston, MA, USA) at 0–4 °C for 60 min. Displacement of [$^3$H]Ro 15-4513 was studied in the presence of 10 μM diazepam (Orion, Espoo, Finland; dissolved in dimethylsulphoxide). Nonspecific binding was determined with 10 μM flumazenil (dissolved in dimethylsulphoxide; Hoffmann-La Roche, Basle, Switzerland). After incubation, the sections were washed in ice-cold incubation buffer three times for 15 s. Sections were then dipped into distilled water, air-dried at RT and exposed with a plastic $^3$H-standard to BioMax MR films for 2 months.

[$^{35}$S]TBPS binding assay was modified from the standard assay (Sinkkonen *et al*., 2001). Sections were preincubated in ice-cold buffer containing 50 mM Tris/HCl (pH 7.4) supplemented with 120 mM NaCl for 15 min. Final incubation in preincubation buffer was done with 3 nM [$^{35}$S]TBPS (PerkinElmer Life Sciences, 193 d.p.m./μL) at RT for 90 min. To study [$^{35}$S]TBPS binding in young male mice (16–20 days old, similar to electrophysiology, see below) 6 nM [$^{35}$S]TBPS (866 d.p.m./μL) was used. Displacement of [$^{35}$S]TBPS binding was studied in the presence of 1 mM GABA, and its modulation with 300 μM furosemide (dissolved in 0.1 N NaOH). After incubation the sections were washed three times for 30 min in ice-cold 10 mM Tris/HCl (pH 7.4). Sections were then dipped into distilled water, air-dried at room temperature and exposed with a plastic $^{14}$C-standard to BioMax MR films for 3 days to 7 weeks. Nonspecific binding was determined with 100 μM picrotoxinin.

Regional labelling intensities of the sections were quantified from the films by using AIS image analysis devices and programs (Imaging Research, St. Catharines, Ontario, Canada). The binding values are given as radioactivity levels estimated for grey matter areas (nCi/mg for [$^3$H]Ro 15-4513 assay; nCi/g for [$^{35}$S]TBPS assay). The specific binding values were determined by subtracting the nonspecific binding values from the corresponding binding values under each incubation condition.

### *Preparation of hippocampal membranes for analysis of subunit proteins*

Receptor extracts were prepared from a total of 30 male control and ten male Thy1α6 mice. Pools containing ten or five hippocampi of control or Thy1α6 mice, respectively, were suspended in 20 mL of a deoxycholate buffer [0.5% deoxycholate, 0.05%, phosphatidylcholine,

10 mM Tris/HCl (pH 8.5), 150 mM NaCl, 1 Complete Protease Inhibitor Cocktail Tablet (Roche Molecular Biochemicals, Mannheim, Germany) per 50 mL]. The suspension was homogenized by using an Ultra-Turrax, subsequently by pressing the suspension through a set of needles with increasingly smaller diameters using a syringe, followed by incubation under intensive stirring for 1 h at 4 °C. After centrifugation at 150 000 ***g*** for 45 min the clear supernatant was used either for subsequent immunoprecipitation and [$^3$H]muscimol binding studies or for affinity chromatography and subsequent immunoprecipitation and [$^3$H]muscimol binding studies in the column efflux.

### *Quantification of GABA$_A$ receptors by immunoprecipitation and [$^3$H]muscimol binding assays*

All experiments were performed in quintuplicate. For immunoprecipitation of all $GABA_A$ receptors present in the hippocampal extracts, 175 or 200 μL of the clear supernatant or immunoaffinity column efflux (see below) was mixed with a solution containing 5 μg α1(1–9), 10 μg β1(350–404), 5 μg β2(351–405) and 5 μg β3(345–408) antibody. This antibody composition was used because all functional $GABA_A$ receptors are supposed to contain at least one of the three β subunits, and most of them contain an α1 subunit (Tretter *et al*., 1997; Pöltl *et al*., 2003). Additionally, the same volume of column efflux was mixed with 15 μg γ2(319–366), 5 μg δ(1–44), 10 μg α6(317–371) or 5 μg α4(379–421) antibody in order to precipitate receptors containing the respective subunit (Pöltl *et al*., 2003). The mixture was then incubated under gentle shaking at 4 °C overnight. Then 50 μL 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, 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, 0.5% Triton X-100, pH 8.3) and once with 500 μL IP-low.

The precipitated receptors were then suspended in 1 mL of a solution containing 0.1% Triton X-100, 50 mM Tris/citrate buffer (pH 7.1) and 40 nM [$^3$H]muscimol (29.5 Ci/mmol, PerkinElmer Life Sciences) in the absence or presence of 1 mM GABA. After incubation for 1 h 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 (Filter-Count, Packard Bioscience, Meridian, CT, USA; 2100 TR Tri-Carb Scintillation Analyser, Packard Bioscience). Binding in the presence of 1 mM GABA (nonspecific binding) was then subtracted from binding in the absence of GABA (total binding), resulting in specific binding to precipitated $GABA_A$ receptors.

### *Immunoaffinity chromatography*

Immunoaffinity columns were prepared by coupling 3–5 mg of the purified antibodies γ1(320–411), γ2(319–366), γ3(322–372) or δ(1–44) to 1 mL of ImmunoPure immobilized protein A using the ImmunoPure Protein A IgG Orientation Kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. The column was then washed once with PBS/NaCl (150 mM NaCl, 4 mM $KH_2PO_4$, 16 mM $Na_2HPO_4$, pH ~7.5), then with glycine-elution buffer, pH 2.45 (0.1 M glycine/HCl, pH 2.45, 150 mM NaCl, 0.1% Triton X-100), with IP-low buffer (see above), with alkaline elution buffer (100 mM $Na_2HPO_4$, 150 mM NaCl at pH 11.5) and finally with IP-low. Columns were stored protected from light in IP-low buffer containing sodium azide. Immunoaffinity chromatography was performed at 4 °C. The immunoaffinity columns were equilibrated in deoxycholate extraction buffer. The extract was chromatographed slowly once or twice on the same affinity column in order completely to remove the receptors containing the respective subunit from the extract, regenerating the respective column after each chromatography step by washing it with glycine-elution buffer (pH 2.45), IP-low (see above), antibody elution buffer (pH 11.5; see above) and IP-low. To determine the percentage of receptors containing a specific subunit or the total amount of receptors retained by the columns, immunoprecipitations with subunit-specific antibodies or with an antibody mixture containing antibodies β1(350–404), β2(351–405), β3(345–408) and α1(1–9) and subsequent [$^3$H]muscimol binding assays were performed in the original extract and column efflux in parallel (see above).

### *Electrophysiological recordings*

Electrophysiological experiments were performed as previously described (Möykkynen *et al*., 2003) using a whole-cell patch-clamp method (Hamill *et al*., 1981). Male mice at the age of 16–20 days ($n = 3$ and 6 for control and Thy1α6 mice, respectively) were decapitated and brains taken into the ice-cold cutting solution (containing in mM: sucrose, 194; NaCl, 30; KCl, 4.5; $MgCl_2$, 1; $NaHCO_3$, 26; $NaH_2PO_4$, 1.2; glucose 10, bubbled with 95% $O_2$/5% $CO_2$ to achieve a of pH 7.4). One brain per day was attached to the platform of a vibroslice cutter (Camden Instruments Ltd, Leicester, UK) and 300–400-μm-thick coronal brain slices were cut. Slices were kept at room temperature in artificial CSF (aCSF) (containing in mM: NaCl, 124; KCl, 4.5; $MgCl_2$, 1; $NaHCO_3$, 26; $NaH_2PO_4$, 1.2; glucose, 10; $CaCl_2$, 2, bubbled continuously with 95% $O_2$/5% $CO_2$). Hippocampal CA1 neurons were isolated after first incubating coronal slices in pronase (Calbiochem, San Diego, CA, USA; 0.4–0.6 mg/mL) in aCSF at 37 °C for 20–30 min. Slices were then transferred to trituration buffer (containing in mM: NaCl, 20; *N*-methyl-D-glucamine, 130; KCl, 2.5; $MgCl_2$, 1; HEPES, 10; glucose, 10, osmolarity adjusted with sucrose to 340 mOsm, pH adjusted to 7.4 with HCl), and the hippocampal CA1 region was cut free of the rest of the tissue. The CA1 neurons were dissociated by gentle mechanical trituration with fire-polished Pasteur pipettes. Cells were then allowed to settle to the bottom of a 35-mm-diameter culture dish, which was then transferred to the stage of an inverted microscope (Leica DM IRB, Leica Mikroskopie und Systeme GmbH, Wetzlar, Germany), under which the patch-clamp recordings were carried out. During the recording the cells were constantly superfused with recording solution (containing in mM: NaCl, 150; KCl, 2.5; $CaCl_2$, 2.5; $MgCl_2$, 1; HEPES, 10 and glucose, 10, pH adjusted to 7.4 with NaOH). GABA, 100 μM picrotoxinin [dissolved in EtOH (16 mM final concentration)] and 300 μM furosemide (dissolved in 0.1 N NaOH, pH adjusted to 7.4 with HCl) were diluted in a recording solution and applied to the cells with a multibarrel fast solution application system (Warner Instrument Corp., Hamden, CT, USA). Patch-clamp electrodes were pulled from glass capillaries manufactured by WPI (Sarasota, FL, USA) with a micropipette puller (Model P-80; Sutter Instrument Co., Novato, CA, USA). Electrodes had a resistance 5–9 MΩ, when filled with internal solution (containing in mM: CsCl, 140; NaCl, 4; $CaCl_2$, 0.5; $MgCl_2$, 4; EGTA, 5; HEPES, 10, pH adjusted to 7.3 with CsOH). Cells were whole-cell patch-clamped at a holding potential of −60 mV. Currents evoked by 1 μM GABA application were recorded using an Axopatch 2B amplifier and pClamp 8.0 software package (Axon Instruments,

Inc., Union City, CA, USA), filtered with a 1-kHz low pass filter and digitized at 10 kHz. GABA application lasted 20 s with 5 s antagonist application in the middle of GABA application. Current amplitudes were measured from the peak of the current trace using pClamp 8.0 software.

### *Observational analysis of the mice*

Twenty-one control mice (ten male, 11 female) and 22 Thy1α6 mice (ten male, 12 female) were tested. Modification of primary screen of the SHIRPA protocol (http://www.mgu.har.mrc.ac.uk/mutabase/shirpa_summary.html) was used to analyse physiology and basic neurological and behavioural properties of the mouse lines (Rogers *et al*., 1997; Vekovischeva *et al*., 2004). Mice were observed by bare eye in a viewing glass jar, in an open arena and on a grid floor. The viewing glass jar was a transparent Plexiglas cylinder (15 × 11 cm), where a mouse was evaluated for 45 s and scored for body position, spontaneous activity, respiratory rate and presence of tremor. The open arena (55 × 33 × 18 cm) floor was covered with a plastic sheet marked with 15 equal squares (11 × 11 cm). The first behavioural reaction of a mouse placed at the centre of the arena (transfer arousal), number of crossed squares, gait, pelvic and tail elevations were assessed for 30 s. The grid floor (40 × 20 cm with 12-mm mesh) was used as a support for the viewing jar and to measure tail suspension, grip strength and negative geotaxis reflex. Negative geotaxis reflex was assessed as a possibility of a mouse to turn around and climb up on the grid floor quickly raised to the vertical plane. The sensorimotor response consisted of pinna, corneal and toe pinch reflexes. Pinna and corneal reflexes were provoked by a stainless steel wire (15 mm in length and 0.15 mm in diameter) with a gently touch on the ear lobe or cornea of the eye. The toe pinch reaction was measured by pinching the central toe of the right hind paw with forceps. A click box generating a sudden tone at 90 dB was held 30 cm above the mouse to measure startle reflex. Touch-escape behaviour was determined as a response to a finger stroke of the back. Struggle-escape behaviour combined the body position, touch-escape response and aggression toward an experimenter and the mouse vocalization provoked by handling.

### *Light–dark test*

Male and female control and Thy1α6 mice ($n = 5$ in each group) were tested. The light–dark test (Crawley & Goodwin, 1980) was started by placing a mouse in the light compartment of a two-compartment box divided into one dark and one lit area (30 × 30 × 35 cm) with an open door (12 × 9 cm) between. The time before the first crossing to the dark compartment (latency), number of crossing between compartments and the time spent in the light compartment (minus the latency) were recorded (Vekovischeva *et al*., 2004). Testing time was 5 min.

### *T-maze test*

Male and female control and Thy1α6 mice ($n = 5$ in each group) were tested. The T-maze consisted of three arms (length of start and goal stems, 50 cm; width, 10 cm; height, 15 cm). The walls were made of grey acrylic. The maze was equipped with three removable guillotine doors with one separating a 12-cm compartment at the beginning of the start arm, and two other 24-cm compartments at the end of the other arms. The T-maze was cleaned by water and paper towel after each mouse but not between trials of a test session (see below). A T-maze alternation procedure allows us to measure exploratory behaviour in an unbiased way (Gerlai, 1998). It involves location-specific blockade of entries to certain arms of the maze by guillotine doors. The procedure comprised one forced trial followed by choice trials over a period of 10 min. Mice were individually placed in the 12-cm start compartment of the T-maze. After 5 s of confinement, the door was lifted and the mouse was allowed to explore the start arm and one of the goal arms. Entry to the other goal arm was blocked. After this first, 'forced' trial, the mice explored the areas available to them and eventually re-entered the start arm and moved down to the start compartment. When they entered the start compartment they were confined there for 5 s by closing the start compartment door. Before reopening the door, the door blocking one of the goal arms was also lifted. The start compartment door was lifted and the first free choice trial began and the mice could choose between the two goal arms. After choosing and entering one goal arm half way down, the other arm was blocked. The mice eventually returned from the explored goal arm down to the start box again. After being confined there for 5 s, the testing cycle continued with another free choice trial. The umber of free choice trials was calculated as a measure of the level of mouse activity. An important aspect of the procedure was that mice were not allowed to go from the chosen goal arm directly to the opposite goal arm. The doors were open for the subsequent choice trial, which thus presented a novel situation for the mouse at each choice trial. Because the presence of a novel stimulus in the goal arm has been shown to elicit exploration of that goal arm (Crusio & van Abeelen, 1986), the mice were expected to explore the arm that was blocked at the previous trial. Consecutive choices made by the mice were measured, and the overall alternation rate during the 10-min was calculated (0%, no alternation; 100%, alternation at each trial).

### *Chemical convulsions*

Mice were injected intraperitoneally (i.p.) with either methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM; 1, 2 or 3 mg/kg, 0.1 mg/mL, dissolved in saline, pH 4; Sigma; $n = 11$ for both male and female control mice; $n = 12$ and 11 for male and female Thy1α6 mice, respectively) or picrotoxinin (5, 7.5 or 15 mg/kg, 0.75 mg/mL, dissolved in saline, pH 5; Sigma, $n = 12$ males and 15 females for both control and Thy1α6 mice). Mice were placed in a transparent Plexiglass cylinder (diameter 7.3 cm), tails were tape-fixed and latency to different convulsions was measured. Picrotoxinin usually resulted in progressive convulsions, where writhing clonus (a brief episode of symmetrical forelimb clonus and a jerk of the neck) was followed by generalized tonic–clonic convulsion followed by tonic hindlimb extensor convulsion. DMCM usually induced only writhing clonus, being followed only sometimes by generalized tonic–clonic convulsion (Kosobud & Crabbe, 1990). Mice were killed by cervical dislocation immediately after observation of generalized tonic–clonic convulsion or at the end of the 15-min observation period, whichever came first. The anticonvulsive potency of tiagabine [1.6, 3.2, 6.4 or 12.8 mg/kg, 0.16–0.64 mg/mL (30 mg/mL tiagabine was dissolved in 1 N NaOH, further diluted to saline, pH adjusted to 7 with HCl); Novo Nordisk, Bagsvaerd, Denmark; $n = 18$ and 21 male and female control mice, respectively; $n = 18$ and 21 for Thy1α6 mice, respectively] against picrotoxinin-induced convulsions was tested with i.p. injection 30 min prior to picrotoxinin administration (7.5 mg/kg i.p.). Latency to convulsions during the 15-min observation period was measured as above.

### *Statistics*

GraphPad Prism (version 3.0; GraphPad Software, San Diego, CA, USA) and SAS-STAT (version 6.11, SAS Institute, Cary, NC, USA) software programs were used. When ANOVA revealed significant differences ($P < 0.05$), it was followed by decreased-factor ANOVA or Dunnett's *post-hoc* tests.

## Results

### *Ectopic expression of α6 subunit leads to formation of hippocampal α6βγ2 and α6β receptors*

To understand better the circumstances behind altered inhibition in the Thy1α6 mice, we extended the previous characterization of ectopic α6 subunit-containing receptors. ISH revealed α6 mRNA expression in Thy1α6 mouse forebrain (Fig. 1), with the highest expression in the hippocampal CA1 region. The hippocampal dentate gyrus and CA3 area, deep layers of neocortex and amygdala also had clear expression.

To image the α6 subunit-containing $GABA_A$ receptors in the forebrain of Thy1α6 mice, ligand autoradiography with protocols revealing two different α6-receptor populations was applied. The γ2 subunit-dependent total [$^3$H]Ro 15-4513 binding was similar in both mouse lines (Fig. 1), except for the CA1 area, where the Thy1α6 mice had 25% more binding (64 ± 1 vs. 80 ± 4 nCi/mg for control and Thy1α6 mice, respectively; mean ± standard error, $n = 5$, $P = 0.0045$, unpaired *t*-test). Diazepam-insensitive (DIS) [$^3$H]Ro 15-4513 binding reveals the α6βγ2 receptors (Turner *et al*., 1991; Mäkelä *et al*., 1997). Diazepam given at 10 μM failed to displace 59 ± 6% of the basal binding in the CA1 area of Thy1α6 mice, whereas in the control mice, the binding was displaced completely from the forebrain, but remained in the cerebellar granule cell layer because of the native α6 subunit (Fig. 1). These results demonstrate that α6 subunits are incorporated in many of the γ2-containing receptors in the CA1 region of Thy1α6 mice.

[$^{35}$S]TBPS binds to picrotoxinin-sensitive sites of all $GABA_A$R ionophores. [$^{35}$S]TBPS autoradiography showed similar basal [$^{35}$S]TBPS binding throughout the brain of both mouse lines (Fig. 1), but the CA1 area of Thy1α6 hippocampus had 28% less binding (335 ± 13 vs. 240 ± 9 nCi/g, for control and Thy1α6 mice, respectively, $n = 7$ and 8, $P < 0.001$, unpaired *t*-test). Because only one low ligand concentration was used, it is not possible to conclude whether this difference is due to reduced receptor number in the CA1 region or due to reduced affinity in α6 subunit-containing receptors, although the apparent $K_D$ constants are similar between the α6 and α1 subunit-containing receptors (Korpi & Lüddens, 1993). Adding 1 mM GABA resulted in a typical GABA-insensitive (GIS) [$^{35}$S]TBPS binding pattern in the control mice, as [$^{35}$S]TBPS was displaced virtually from all forebrain regions except the thalamus (Sinkkonen *et al*., 2001). The remaining GIS-[$^{35}$S]TBPS binding activity (note the longer exposure times under conditions where 1 mM GABA inhibits the binding) represents 0.7 ± 0.1% of the binding in the absence of GABA in the hippocampal CA1 region. Strikingly, in Thy1α6 brains, in addition to its normal distribution, GIS-[$^{35}$S]TBPS binding was clearly visible in the CA1 region, where 8.9 ± 0.4% of basal binding was not abolished by GABA. It has been demonstrated previously (Korpi & Lüddens, 1993; Sinkkonen *et al*., 2004) that GABA-insensitive TBPS binding can also be produced by α6β2/3 receptors, whereas α6β2/3γ2 receptors exhibit normal GABA-sensitivity of [$^{35}$S]TBPS binding. The residual binding found in Thy1α6 brains, thus represents α6β receptors. When the α6-selective antagonist furosemide (Korpi *et al*., 1995) was incubated at 300 μM together with 1 mM GABA, it antagonized the inhibitory effect of GABA in the hippocampus and deep layers of neocortex of the Thy1α6 mice, but had no effect in the control mice (Fig. 1), again indicating the presence of α6-$GABA_A$Rs.

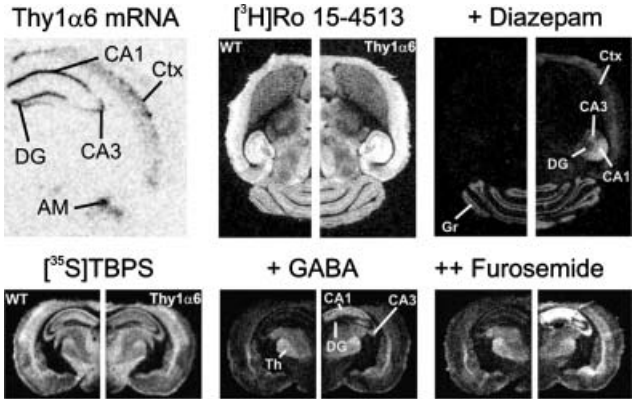


FIG. 1. Distribution of Thy1α6 mRNA expression and [$^3$H]Ro 15-4513 and [$^{35}$S]TBPS binding sites in control and Thy1α6 mouse brain sections. [$^3$H]Ro 15-4513 binding was studied with and without 10 μM diazepam. [$^{35}$S]TBPS was studied with and without 1 mM GABA in the absence or additional presence of 300 μM furosemide. AM, amygdala; CA1 and CA3, respective hippocampal areas; Ctx, cortex; Gr, cerebellar granule cell layer; DG, dentate gyrus; Th, thalamus. Exposure times of film images, scanned with identical scaling for brightness and contrast, were 3 days for the basal [$^{35}$S]TBPS and 7 weeks for films in all other conditions.

In the previous study it was demonstrated that most α6-$GABA_A$Rs in whole forebrain extracts of Thy1α6 mice contained γ2, but not δ, subunits (Wisden *et al*., 2002). Because we detected increased GIS-[$^{35}$S]TBPS binding in the hippocampal CA1 region suggestive for α6β receptors (Korpi & Lüddens, 1993; Sinkkonen *et al*., 2004), we wanted to determine the subunit composition of the hippocampal $GABA_A$ receptors in the control and Thy1α6 mice. $GABA_A$ receptors were extracted from control and Thy1α6 hippocampi and extracts were applied on subsequent affinity columns containing antibodies directed against γ1, γ2, γ3 and δ subunits. To determine the total amount of receptors and the percentage of receptors containing a specific subunit in the efflux of the columns, immunoprecipitation with an antibody mixture (α1, β1, β2, β3; see Materials and methods) or with subunit-specific antibodies and subsequent [$^3$H]muscimol binding assays were performed in the original extract and in column efflux in parallel (Fig. 2). Immunoprecipitations from the column efflux of control animals indicated that all the receptors still present in the efflux (99 ± 9%) contained γ2 subunits, suggesting that the anti-γ2 column could not completely retain all γ2-containing receptors. As no γ1, γ3 or δ subunit-containing receptors could be identified in this efflux, these data indicate that no significant amounts of αβ receptors could be detected in the hippocampus of control mice. The majority of receptors in the efflux presumably contained α1, α2, α3 or α5 subunits, because no significant amounts of α4 or α6 subunit-containing receptors could be detected. Receptors present in the Thy1α6 column efflux in two independent experiments contained only 61 and 56% γ2 subunits and no γ1, γ3 or δ subunits, thus indicating the existence of 9 and 12% αβ receptors in the whole hippocampus of these mice (amounting to 39 and 44% of the receptors in the column efflux, respectively). Furthermore, 30 and 7%, or 70 and 81% of all receptors found in the column efflux of Thy1α6 mice contained α4 or α6 subunits, respectively, thus suggesting that most, if not all, αβ receptors contained α4 or α6 subunits.

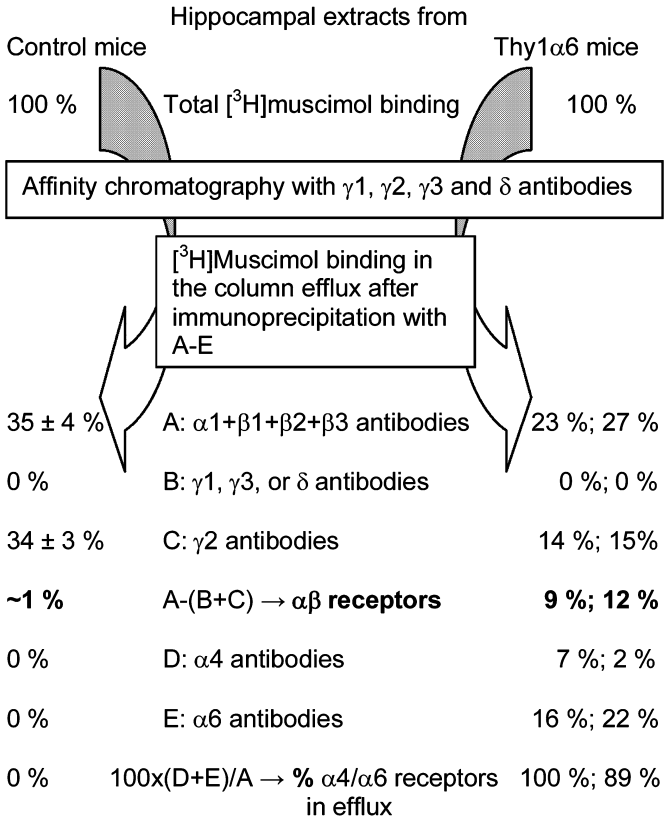


FIG. 2. Percentage of $GABA_A$ receptor αβ subtypes present in the hippocampal extracts of control and Thy1α6 mice. $GABA_A$ receptors were extracted from the hippocampi of control or Thy1α6 mice and the extracts were chromatographed on subsequent affinity columns containing antibodies directed against γ1, γ2, γ3 and δ subunits. To determine the percentage of the total amount of receptors or of receptors containing a specific subunit in the efflux of the columns, immunoprecipitation with an antibody mixture or with subunit-specific antibodies and subsequent [$^3$H]muscimol binding assays were performed in the original extract and in column efflux in parallel. All extracts were analysed in quintuplicates, and the data are given as mean ± standard error ($n = 3$) for control mice and as means from two separate experiments for Thy1α6 mice. The data demonstrate that the Thy1α6 mice have about 10% of their hippocampal $GABA_A$ receptors as αβ combinations, and that most of these receptors seem to contain α6 or/and α4 subunits.

### *Ectopic α6 subunits form functional receptors in the Thy1α6 hippocampal CA1 pyramidal cells*

To probe whether the ectopic α6-containing receptors in the Thy1α6 mice are functional, whole-cell patch clamp on acutely isolated CA1 pyramidal cells was applied. When recording the whole cell currents from isolated cells, total current may represent both synaptic and extrasynaptic receptors. As the proposed α6βγ2 and α6β receptors in the Thy1α6 mice are extrasynaptic (Wisden *et al*., 2002), they should be activated *in vivo* by low ambient GABA. To mimic this, 1 μM GABA was applied. The subtype-nonselective $GABA_A$ receptor antagonist picrotoxinin blocked the GABA-induced current in both control and Thy1α6 cells as expected (Fig. 3). However, 300 μM furosemide affected currents only in cells from mutant mice (Fig. 3), inhibiting 55 ± 17% (mean ± standard error, $n = 14$) of the 1 μM GABA-induced currents. This is the first direct evidence for functional expression of α6-$GABA_A$Rs in the Thy1α6 mice. In the immunohistochemical studies, only some cells were α6-immunopositive in the brain regions of Thy1α6 transgene expression (Wisden *et al*., 2002). We confirmed this by finding that only 14 out of 40 (35%) isolated

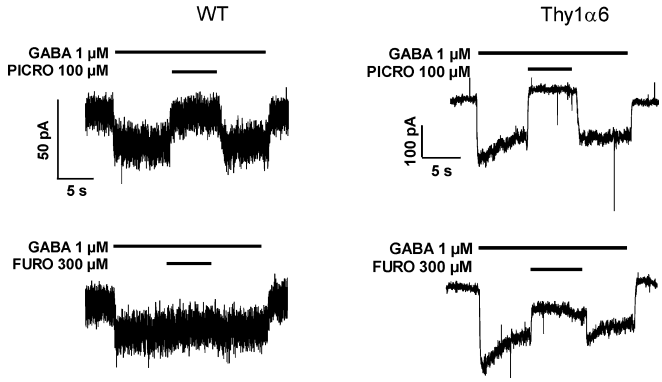


FIG. 3. Inhibition of GABA-induced currents by picrotoxinin and furosemide in isolated hippocampal CA1 pyramidal neurons from control and Thy1α6 mice. Picrotoxinin (PICRO 100 μM; upper row) completely blocked 1 μM GABA-induced currents in cells from both control ($n = 6$) and Thy1α6 ($n = 40$) mice, whereas 300 μM furosemide (FURO) produced a partial effect only on some (35%) mutant cells (lower row), inhibiting 55 ± 17% (mean ± standard error, $n = 14$) of the GABA-induced currents. Note that the larger current responses shown for the neurons from Thy1α6 mice were most likely due to ectopic expression of highly GABA-sensitive α6 subunit-containing receptors.

Thy1α6 CA1 pyramidal cells were furosemide-sensitive. Mice used in electrophysiological recordings were young (16–20 days old), but all our other experiments were performed with adult animals. To control the validity of our comparison between electrophysiological data and data from other experiments, brain sections from four young Thy1α6 mice and one young control mouse (that were also used for electrophysiological recordings) were tested via [$^{35}$S]TBPS autoradiography, and the results between the genotypes were identical to those for the adult mice (data not shown).

### *Thy1α6 mice display largely normal basic behavioural phenotype*

Thy1α6 mice look healthy, and grow and breed normally. To test the behavioural significance of the changed balance between synaptic and extrasynaptic inhibition, male and female control and Thy1α6 mice were used in behavioural assays. In the basic behavioural assessment (Rogers *et al*., 1997; Vekovischeva *et al*., 2004) no gender effects in any of the parameters were detected. Thus, data from both genders were pooled. The Thy1α6 mice displayed grossly normal behaviour in the observational analysis, but differences from control mice were detected in startle reflex and struggle-escape behaviours. In the startle reflex, Thy1α6 mice responded to abrupt sounds more actively (Fig. 4A; $F_{1,43} = 68.08$, $P < 0.001$, one-way ANOVA), because acoustic stimuli usually produced a jumping response. Mutant mice also had enhanced struggle-escape behaviour (Fig. 4A; $F_{1,43} = 4.55$, $P = 0.039$, one-way ANOVA), suggesting heightened reactivity towards the observer.

Next, we used a light–dark test (Crawley & Goodwin, 1980) to assess hippocampus- and amygdala-dependent anxiety-like behaviour in the male and female control and Thy1α6 mice ($n = 5$ in each group). Two-way ANOVA revealed a gender effect ($F_{1,29} = 8.47$, $P = 0.0102$), which was due to control females making more crossings between the compartments than control males (24.4 ± 1.4 vs. 14.2 ± 2.9, $F_{1,10} = 10.00$, $P = 0.013$). The Thy1α6 mice did not show a gender difference in the number of crossings, nor differences when compared with controls ($F_{1,20} = 1.36$, for factor of mouse line, n.s.). The latency to the first entry into the dark compartment and total

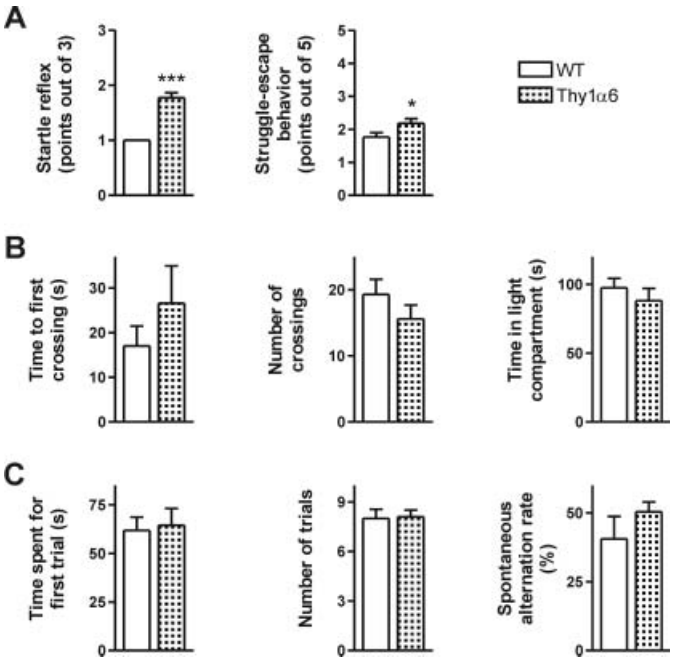


FIG. 4. Behavioural characterization of control and Thy1α6 mice. (A) Startle reflex and struggle-escape behaviour were enhanced in the mutant mice. Data are mean ± standard error, $n = 21$ and 22 for control and Thy1α6 mice, respectively. $^{*}P = 0.0367$ and $^{***}P < 0.001$ for the significance of the difference, one-way ANOVA. (B) Unchanged anxiety-like behaviour between control and Thy1α6 mice in the light–dark test. Data are mean ± standard error, $n = 10$ for both mouse lines. (C) Unchanged T-maze behaviour between control and Thy1α6 mice. Data are mean ± standard error, $n = 10$ for both mouse lines.

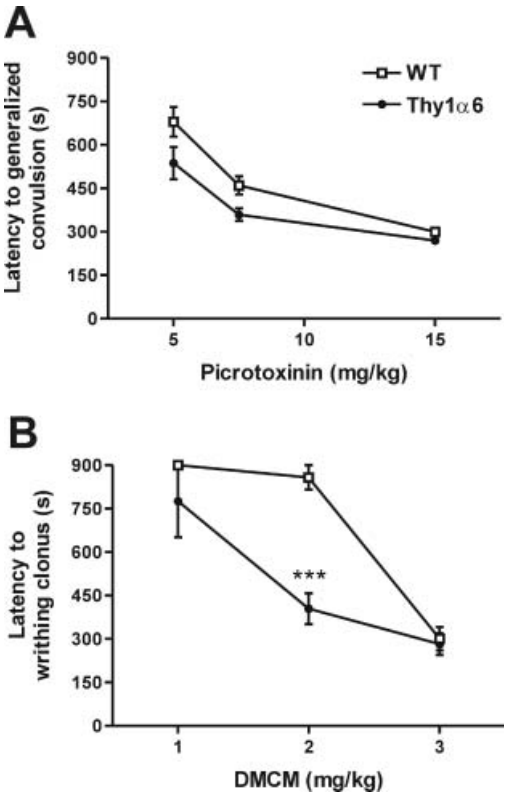


FIG. 5. Proconvulsant effects of $GABA_A$ receptor antagonists in control and Thy1α6 mice. (A) Latency to generalized convulsions induced by intraperintoneal injection of picrotoxinin. Data are mean ± standard error, $n = 8$–9. Latency to convulsions was shorter in Thy1α6 mice ($F_{1,54} = 16.39$ for factor of mouse line, $P = 0.0002$, three-way ANOVA), but there were no significant differences at any picrotoxinin dose between mouse lines in one-way ANOVA. (B) Latency to writhing clonus induced by intraperintoneal injection of DMCM. Data are mean ± standard error, $n = 6$–10. Latency was significantly shorter in Thy1α6 mice ($F_{1,46} = 12.74$ for factor of mouse line, $P = 0.0011$, three-way ANOVA). The difference was statistically significant at a dose of 2 mg/kg ($^{***}P < 0.0001$, one-way ANOVA).

time spent in the light compartment were similar in both mouse lines (Fig. 4B; $F_{1,20} < 0.78$, n.s.), all these data indicating unaltered background anxiety in the Thy1α6 mice. Next, we applied the T-maze test in a continuous alternation task protocol (Gerlai, 1998). Both male and female mice of both lines were used ($n = 5$ in each group). No gender differences were observed. Between the mouse lines, no differences in time spent for the first forced trial or number of choice trials were detected (Fig. 4C; $F_{1,20} < 0.3$, n.s.). Spontaneous alternation rate was similar ($F = 1.44$, n.s.) in both mouse lines.

### *Altered seizure sensitivity in Thy1α6 mice*

On the basis of the pronounced startle reflex and struggle-escape behaviour (Fig. 4A) in the presence of otherwise normal behaviour, we suspected that the altered balance between synaptic and extrasynaptic inhibition in Thy1α6 mice would affect behavioural responses caused only by strong stimuli. Therefore, we studied the susceptibility of the Thy1α6 mice to seizure induction by $GABA_A$R antagonists. First, both male ($n = 12$ for both control and Thy1α6) and female ($n = 15$ for both control and Thy1α6) mice were tested for convulsive responses to picrotoxinin (Gibbs *et al.*, 1997). No gender differences in the picrotoxinin effect were observed. Thy1α6 mice had significantly shorter latencies to generalized convulsions (Fig. 5A; $F_{1,54} = 16.39$, $P = 0.0002$). Picrotoxinin decreased the latency to convulsions in a dose-dependent manner ($F_{2,54} = 74.60$, $P < 0.0001$). When the data from both genders were pooled and the effect of different picrotoxinin doses was compared between the mouse lines using one-way ANOVA, the differences did not reach significance ($P > 0.05$). Next, we studied the sensitivity to the potent inverse agonist DMCM, which has proconvulsant actions via antagonism of α1 subunit-containing receptors (Crestani *et al.*, 2002). It also has partial agonistic effects on α6 subunit-containing receptors (Knoflach *et al.*, 1996; Saxena & Macdonald, 1996). Intraperitoneal injections of DMCM resulted in writhing clonus in both mouse lines ($n = 11$ for both control males and females; $n = 12$ and 11 for Thy1α6 males and females, respectively), and no gender differences were detected. Thy1α6 mice had a shorter latency to convulsions (Fig. 5B; $F_{1,46} = 12.74$, $P = 0.0011$). In addition, the DMCM dose effect and the interaction between DMCM dose and mouse line were statistically significant ($F_{2,46} = 36.59$, $P < 0.0001$ and $F_{2,46} = 5.49$, $P = 0.0086$, respectively). When the data for both genders were pooled and the effects of different DMCM doses were compared using one-way ANOVA, the difference in the latency to convulsions after DMCM injections was statistically significant at a dose of 2 mg/kg ($P < 0.0001$). At this DMCM dose only one of seven control mice had a convulsion during the 900-s observation period, whereas all Thy1α6 mice tested ($n = 7$) displayed writhing clonus. This indicates that Thy1α6 mice are more susceptible to convulsions induced by the inverse agonism of α1βγ2 receptors, and that this effect cannot be counteracted by the partial agonist effect of DMCM on extrasynaptic α6βγ2 receptors in the Thy1α6 mice.

### *Increasing tonic inhibition* in vivo *delays convulsions but cannot fully compensate for decreased phasic inhibition*

In the last experiment we tested more directly whether an increase in extracellular GABA by GABA uptake blockade is more effective in reversing the generalized convulsions induced by picrotoxinin in the Thy1α6 than in control animals. In the Thy1α6 mice, a tiagabine-derivative (NO-711) revealed *in vitro* a five-fold increased background conductance in the CA1 pyramidal cells (Wisden *et al*., 2002). The GABA-uptake blocker tiagabine delays chemically induced convulsions (Dalby, 2000). Here we demonstrate that low doses of tiagabine had virtually no effects on animal behaviour, but both mouse lines showed similar sedation (loss of exploration) after 6.4 and 12.8 mg/kg tiagabine injections. Thirty minutes after tiagabine, picrotoxinin (7.5 mg/kg i.p.) was given. Both male ($n = 18$ and 24 for control and Thy1α6 mice, respectively) and female ($n = 21$ and 22 for control and Thy1α6 mice, respectively) mice were tested. Differences in the action of tiagabine were not found between genders. Then the tiagabine effects on both mouse lines were analysed separately by two-way ANOVA (factors: gender, tiagabine dose). In control mice tiagabine delayed generalized convulsions dose-dependently ($F_{4,48} = 14.40$, $P < 0.0001$), the effect being statistically significant at doses of 6.4 and 12.8 mg/kg (Dunnett's *post-hoc* test) when compared with control values (latency to convulsion after 7.5 mg/kg picrotoxinin alone; Fig. 6). The lower tiagabine doses were ineffective (Fig. 6). In the Thy1α6 mice, tiagabine had dose-dependent anticonvulsant action ($F_{4,55} = 12.86$, $P < 0.0001$), the effect being statistically significant even at a dose of 3.2 mg/kg (Dunnett's *post-hoc* test; Fig. 6), and remained effective at higher doses. When tiagabine effects of both mouse lines were compared (three-way ANOVA (factors: gender, mouse line, tiagabine dose), tiagabine was more effective in the control mice (Fig. 6; $F_{1,103} = 17.21$, $P < 0.0001$ for mouse line; $F_{4,103} = 23.21$, $P < 0.0001$ for tiagabine dose; $F_{4,103} = 2.68$, $P = 0.037$ for interaction between mouse line and tiagabine dose). The mouse lines differed in their latencies to convulsions at 6.4 and 12.8 mg/kg tiagabine ($P < 0.001$ for both concentrations, one-way ANOVA). After the highest tiagabine dose, only one control mouse out of seven proceeded to generalized convulsion during the 900-s observation period, whereas nine out of 11 Thy1α6 mice had convulsions. Together, these results demonstrate that a low dose (3.2 mg/kg) of tiagabine had anticonvulsive activity in Thy1α6, but not in control mice, whereas the overall efficacy of tiagabine against picrotoxinin-induced convulsions in the Thy1α6 mice was decreased.

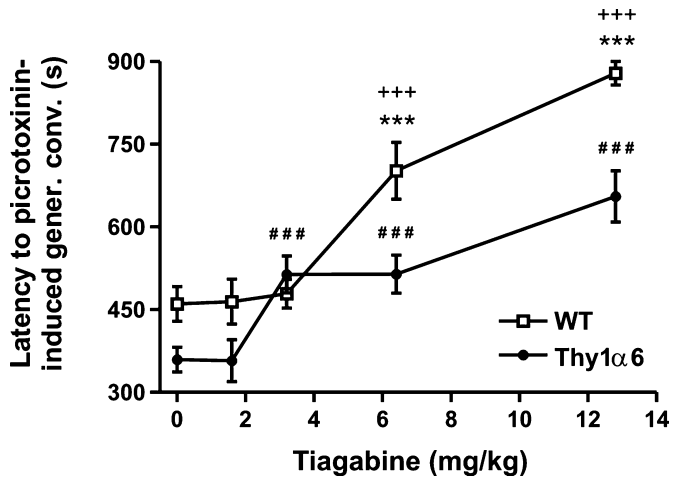


FIG. 6. Anticonvulsant effect of tiagabine against picrotoxinin-induced generalized convulsions in control and Thy1α6 mice. Mice were pretreated with different tiagabine doses 30 min prior to picrotoxin injection (7.5 mg/kg, i.p.). Data are mean ± standard error, $n = 7$–16. Tiagabine increased the latency to convulsions in control mice [$F_{4,48} = 14.40$, $P < 0.0001$, two-way ANOVA (factors: gender and tiagabine dose)]; the effect was significantly different from the control value (7.5 mg/kg picrotoxinin alone) at doses of 6.4 and 12.8 mg/kg (***$P < 0.001$, Dunnett's *post-hoc* test). In the Thy1α6 mice, tiagabine increased the latency to convulsions ($F_{4,48} = 12.86$, $P < 0.0001$, two-way ANOVA), the effect being significant at doses of 3.2, 6.4 and 12.8 mg/kg ($^{###}P < 0.001$, Dunnett's *post-hoc* test). When tiagabine effects of both mouse lines were compared [three-way ANOVA (factors: gender, mouse line, tiagabine dose)], tiagabine was more effective in the control mice ($F_{1,103} = 17.21$, $P < 0.0001$ for factor of mouse line; $F_{4,103} = 23.21$, $P < 0.0001$ for factor of tiagabine dose; $F_{4,103} = 2.68$, $P = 0.037$ for interaction of mouse line and tiagabine dose factors), the difference being statistically significant at doses of 6.4 and 12.8 mg/kg ($^{+++}P < 0.001$, one-way ANOVA).

## Discussion

The Thy1α6 mouse line with a changed balance between synaptic and extrasynaptic $GABA_A$ergic inhibition especially in the hippocampal CA1 region (Wisden *et al*., 2002) had an interesting albeit subtle behavioural phenotype, without any evidence for drastically compromised hippocampal function. The proportion of extrasynaptic receptors in the CA1 region of the Thy1α6 mice may be maximally about 60% on the basis of α6βγ2 subtype-selective autoradiography (DIS-[$^3$H]Ro 15-4513 binding, which overestimates the α6 receptors due to their higher affinity) and minimally 9% on the basis of α6β subtype-selective autoradiography (GIS-[$^{35}$S]TBPS binding, which underestimates the α6 receptors because GABA partially displaces the ligand from them). Both assays failed to show any clear signal in the control brains. Immunoprecipitation experiments on whole hippocampi revealed about 10% of total $GABA_A$ receptors being αβ (mostly α6) in the Thy1α6 mice (Fig. 2). Taking into account these autoradiographic and immunoprecipitation results and if we assume that about one-third of the neurons expressed the transgene [furosemide-sensitive currents in 35% of the isolated CA1 neurons (Fig. 3) and immunohistochemical data of transgene expression (Wisden *et al*., 2002)], we can estimate that at least 30% of the $GABA_A$ receptors in the Thy1α6 mice are extrasynaptic in the hippocampal CA1 region. Some neurons may thus be more affected than others. However, the effects of the ectopic receptors might still be small also because in extrasynaptic α6β receptors GABA acts only as a partial agonist (Korpi & Lüddens, 1993; Sinkkonen *et al*., 2004).

In the basic behavioural assessment the mutant mice showed enhanced startle reflex and struggle-escape behaviour (Fig. 4A). The auditory startle reflex is a protective reaction mediated by a brain-stem circuit and modulated by forebrain areas such as the amygdala (Koch, 1999). The hippocampus in turn may modulate the startle reflex via projections from the ventral hippocampus to the amygdala (Pitkänen *et al*., 2000). The α6 transgene is expressed both in the amygdala and in the hippocampus of Thy1α6 mice (Fig. 1). Struggle-escape behaviour involves complex behavioural responses and cannot be assigned to any particular brain region. Regardless, it also suggests slightly increased behavioural excitability of the Thy1α6 mice.

Anxiety-like behaviour is relieved by $GABA_A$ergic drugs, and deficits in the $GABA_AR$ system in the hippocampus are reported in panic disorder patients (Kaschka *et al*., 1995). In an animal model of human anxiety ($GABA_AR$ $\gamma2^{+/-}$ mice), the phenotype may result from reduced synaptic clustering of $GABA_ARs$ and diminished synaptic inhibition (Crestani *et al*., 1999). An enhanced startle reflex has been detected in another mouse model of increased anxiety, the

BALB mice (Plappert & Pilz, 2002). The fact that the Thy1α6 mice showed no anxiety-like behaviour in the light–dark test (Fig. 4B) indicates that these mice do not have such widespread and strong deficits in $GABA_AR$ function that are seen in the anxious $\gamma2^{+/-}$ mice. The Thy1α6 mice had normal hippocampal performance also in the T-maze test (Fig. 4C), which is used to reveal impaired exploratory behaviour, learning and memory (Gerlai, 1998). Thus, these basic behavioural experiments on anxiety, exploration and memory revealed no changes between mouse genotypes, suggesting that the alteration in the balance between tonic and phasic inhibitions can be functionally well tolerated. However, when we challenged the Thy1α6 mice with the benzodiazepine site-negative allosteric modulator (inverse agonist) DMCM, which produces convulsions by antagonizing solely the α1βγ2 subunit-containing $GABA_ARs$ (Crestani *et al*., 2002), we found shorter latencies to convulsions in the mutant mice (Fig. 5B). This receptor subtype typically is targeted in the synapses, suggesting that reducing the already decreased synaptic inhibition in the Thy1α6 mice was critical for the early onset of convulsions after DMCM. Interestingly, DMCM may also have positively (agonistically) modulated α6βγ2 receptors (Knoflach *et al*., 1996; Saxena & Macdonald, 1996) in the Thy1α6 mice. This action of DMCM seems to be a minor one and it may not sufficiently counteract the antagonistic effect of DMCM on α1-containing receptors. It should be noted that the reduction of α1-containing $GABA_ARs$ is believed to underlie differences in seizure sensitivity between various mouse models and selectively bred rat lines (Poulter *et al*., 1999; Kralic *et al*., 2002). Furthermore, a point mutation in the human α1 subunit, causing a decrease in receptor function (Fisher, 2004), is associated with hereditary juvenile onset epilepsy (Cossette *et al*., 2002).

Unlike DMCM, the $GABA_AR$ channel blocker picrotoxinin inhibits $GABA_ARs$ nonselectively. We assumed that the latency to convulsions after picrotoxinin injection would reflect the capacity of the whole $GABA_A$ergic system to prevent uncontrolled excitability. Similarly to DMCM, picrotoxinin resulted in shorter latency to generalized convulsions in Thy1α6 mice (Fig. 5A). These data confirm that Thy1α6 mice are more susceptible to convulsions produced by $GABA_A$ receptor antagonists. This greater susceptibility to convulsions results most likely from reduced synaptic $GABA_A$ receptor responses.

We hypothesized that the antiepileptic drug tiagabine would be more protective in the Thy1α6 than in control mice, because elevated ambient GABA levels (Dalby, 2000) would activate more easily the highly GABA-sensitive α6-receptors, as suggested here by electrophysiology of isolated hippocampal CA1 neurons (Fig. 3), and thus cause increased background inhibition similar to the *in vitro* effects seen with another GABA-uptake inhibitor (Wisden *et al*., 2002). Interestingly, tiagabine delayed the picrotoxinin-induced convulsions even at the low dose of 3.2 mg/kg in the Thy1α6 mice (Fig. 6). The same dose did not affect the latency to convulsions in the control mice (Fig. 6). This suggests that tonic $GABA_A$ergic conductance was increased by elevated GABA levels *in vivo*, and demonstrates for the first time a functional correlate of increased background conductance. A schematic presentation of the situation is given in Fig. 7. Nevertheless, higher tiagabine concentrations had an antiepileptic effect also in the control mice, and overall the effect of tiagabine was greater in the control mice (Fig. 6). This might be due to the presence of an atypical α6β receptor population in the Thy1α6 mice, in which population GABA probably has only partial agonist activity (Korpi & Lüddens, 1993; Sinkkonen *et al*., 2004). It remains to be directly tested whether the balance between extrasynaptic and synaptic inhibitions is generally regulated in epileptic processes and during treatments with antiepileptic drugs. Selective modulation of tonic

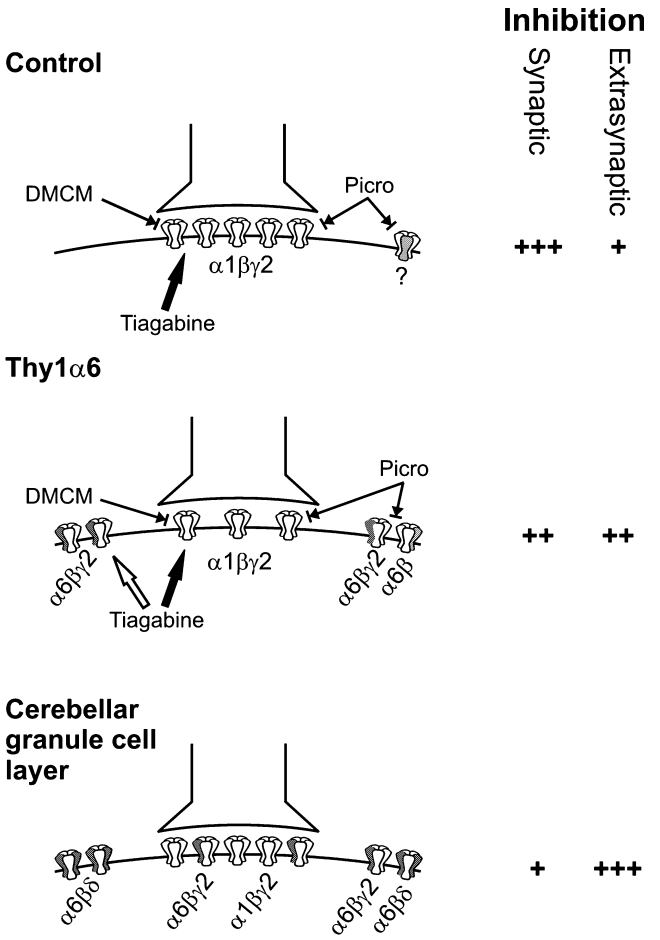


FIG. 7. A schematic presentation of the $GABA_A$ergic system in hippocampal interneuron-pyramidal cell synapses of control and Thy1α6 mice. The number of receptor complexes on synaptic and extrasynaptic locations reflects the amount of inhibition mediated by these receptors. DMCM causes convulsions by inhibiting synaptic α1βγ2 receptors, the function of which has already decreased in the Thy1α6 mice. The decreased miniature IPSC amplitude detected in Thy1α6 CA1 pyramidal cells (Wisden *et al*., 2002) suggests a decreased number of synaptic $GABA_A$ receptors as indicated in the figure. Picrotoxinin (Picro) blocks all synaptic and extrasynaptic receptors. Highly GABA-sensitive α6 subunit-containing receptors in Thy1α6 mice are activated by raised GABA levels after low-dose (3.2 mg/kg) tiagabine administration (open arrow), whereas the action of other receptors needs further increases in GABA concentrations after larger tiagabine doses (closed arrow). ?, extrasynaptic receptors of unknown identitity in the control mice, e.g. α5β2/3γ2 (Caraiscos *et al*., 2004). In the cerebellar granule cells most of the inhibition is of tonic nature, and α6βδ receptors convey the major part of it (lowest panel; Hamann *et al*., 2002).

inhibition might offer a more effective therapy with fewer side-effects, because it might be achieved at lower drug doses. It should also be studied whether modulation of tonic inhibition always causes alterations in synaptic efficacy, as suggested by the results from Thy1α6 mice (Wisden *et al*., 2002), from administrations of the antiepileptic vigabatrin (Overstreet & Westbrook, 2001; Wu *et al*., 2003) and from experiments with GABA transporter GAT-1 knockout mice (Jensen *et al*., 2003).

Our study offers the first hints regarding the behavioural significance of an altered balance between synaptic and extrasynaptic inhibition. Although it demonstrates that increased tonic conductance may be compensated with a decrease in synaptic transmission to maintain normal behaviour, it stresses the importance of a synaptic

component in controlling responses to strong stimuli. Even if the tonic conductance cannot replace phasic responses in situations where a fast and powerful inhibition at synapses is compromised (the result from our DMCM experiment, Fig. 5B), it might have an important role in setting the background inhibitory tone in neuronal circuits and controlling the information flow in selected brain regions (Hamann *et al*., 2002). Importantly, low doses of tiagabine, presumably leading to enhanced tonic conductance, could attenuate the effects of decreased synaptic inhibition. The Thy1α6 mouse line could serve as a useful model in further studies, shedding light on the complex balance between synaptic and extrasynaptic inhibition.

## Acknowledgements

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## Abbreviations

DIS, diazepam-insensitive [$^3$H]Ro 15-4513 binding; DMCM, methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate; GABA, γ-aminobutyric acid; $GABA_AR$, $GABA_A$ receptor; GIS, GABA-insensitive [$^{35}$S]TBPS binding; ISH, *in situ* hybridization; Ro 15-4513, ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo(1,5-a)[1,4]benzodiazepine-3-carboxylate; RT, room temperature; TBPS, *t*-butylbicyclophosphorothionate; Thy1α6, a mouse line expressing $GABA_A$ receptor α6 subunit under Thy-1.2 promoter.

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