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ORIGINAL ARTICLE

Formation of GABA_A receptor complexes containing α1 and α5 subunits is paralleling a multiple T-maze learning task in mice

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Abstract There is limited information on the role of GABA type A receptors (GABA_ARs) containing α1, α5 and γ2 subunits in learning and memory. Here, we assessed the possible role of such receptors in spatial learning using the multiple T-maze (MTM) paradigm. C57BL/6J mice were trained in the MTM which induced elevated levels of α1 and α5 subunit-containing hippocampal GABA_AR complexes. Moreover, spatial learning evoked a significant increase in the colocalization of α1 and α5 subunits in both, CA1 and dentate gyrus regions of the hippocampus suggesting the formation of complexes containing both subunits. Additionally, the presence of α1, α5 and γ2 subunits in high molecular weight GABA_ARs was detected and significant correlation in the level of α1-containing complexes with those containing α5 and γ2 subunits was demonstrated. Accordingly, α1 deficiency led to decreased levels of γ2 subunit-containing complexes, however, had no effect on α5-containing ones. On the other hand, α1 knockout mice showed impaired performance in the MTM correlating with increased levels of α5 subunit-containing GABA_ARs in comparison to trained floxed control animals which quickly learned the task. Taken together, these results suggest that α1, α5 and γ2-containing hippocampal GABA_AR complexes play an essential role in spatial learning and memory in which targeted disruption of the α1 subunit produces profound deficits.

Keywords GABA_A receptor complexes · GABA_AR α1 KO mice · Multiple T-maze · Spatial memory · BN-PAGE · Mass spectrometry

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Introduction

Gamma-aminobutyric acid (GABA) is the most important inhibitory neurotransmitter in the brain and the majority of its actions are mediated via GABA type A receptors (GABA_ARs) (Luscher et al. 2011). GABA_ARs are heteropentameric ligand-gated ion channels, typically composed of two α, two β, and one γ subunit. The binding of GABA to two GABA binding sites at the interface between β and α subunits opens the receptor-associated chloride channel (Uusi-Oukari and Korpi 2010).

GABA_ARs are major participants in neuronal processes and thus, control brain development including proliferation and differentiation of neural progenitor cells, neural migration and dendritic maturation (Luscher et al. 2011). Impairments in functional expression of GABA_ARs are critical in epilepsy, anxiety disorders, cognitive deficits,

schizophrenia, mood disorders, depression and substance abuse making the receptors clinically relevant drug targets (Rudolph and Mohler 2014).

A total of 19 mammalian genes coding for GABA$_A$R subunits that belong to eight classes have been cloned: α1–α6, β1–β3, γ1–γ3, δ, ε, θ, π, ρ1–ρ3 (Olsen and Sieghart 2008). Hippocampus, a major area for memory processes, is known to contain α1, α5 and γ2 subunits (Fritschy and Mohler 1995; Pirker et al. 2000; Gutierrez et al. 1994) and growing evidence exists in the literature for the importance of these subunits in learning and memory.

Mice with targeted genetic deletion of the GABA$_A$R α1 subunit displayed impaired performance in Morris water maze (MWM) tasks (Berry et al. 2008) and spatial learning deficits in rats induced by benzodiazepines are regulated by modulation of α1 subunit-containing GABA$_A$Rs (α1GABA$_A$Rs) (Joksimovic et al. 2013). Other pharmacological and genetic studies have also provided evidence for the role of the α5 subunit in hippocampus-dependent learning (Chambers et al. 2004; Collinson et al. 2002; Crestani et al. 2002; Dawson et al. 2006; Gerdjikov et al. 2008; Yee et al. 2004). Indeed, an inverse agonist selective for the α5 subunit-containing GABA$_A$Rs (α5GABA$_A$Rs) improved encoding and recall but not consolidation in the MWM (Collinson et al. 2006) which reaffirms the role of this subunit in learning and memory. Studies conducted to assess the differential role of α1 and α5 subunit-containing GABA$_A$Rs in learning and memory suggested that whilst explicit memory seems to be mediated through GABA$_A$Rs containing α1 and/or α5 subunits, procedural memory is largely mediated by the α1 subunit (Prut et al. 2010; Savic et al. 2005).

Although some evidence has been provided for the existence of GABA$_A$Rs containing both, α1 as well as α5 subunits (Araujo et al. 1999; Olsen and Sieghart 2008), a possible role of such receptors in learning and memory has not yet been elucidated. Thus, the aim of the current study was to investigate modulation and involvement of α1, α5 and γ2-containing GABA$_A$R complexes in spatial learning and memory formation.

Materials and methods

C57BL/6J mice ($n = 10$) were trained in the multiple T-maze (MTM) and training-induced changes in GABA$_A$R complex levels were analyzed by blue native gel electrophoresis with subsequent western blot analysis (BN-PAGE/WB) from hippocampal membrane fractions ($n = 10$ trained − 10 untrained). Moreover, training-induced changes in the colocalization of α1 and α5 subunits were examined in C57BL/6J mice (a new set of $n = 5$ animals were trained in the MTM) via immunofluorescent

staining and confocal microscopy ($n = 5$ trained − 5 untrained). Independently, the presence of α1, α5 and γ2 subunits in the same high molecular weight GABA$_A$R complex was investigated by two-dimensional BN/SDS-PAGE/WB, immunoprecipitation and liquid chromatography—tandem mass spectrometry (LC–MS/MS). Furthermore, α1 knockout (KO) and floxed control mice ($n = 13$ control − 14 α1 KO) were trained in the MTM and differences in GABA$_A$R complex levels between α1 KO and wild type controls were compared in baseline conditions ($n = 7$ untrained control − 7 untrained α1 KO) as well as following the training ($n = 6$ trained control − 6 trained α1 KO) via BN-PAGE/WB of hippocampal membrane fractions (see experimental outline: Fig. S1).

Animals

C57BL/6J ($n = 30/15$ trained + 15 untrained/; male; aged 10–12 weeks) and GABA$_A$R α1 subunit knockout (KO) mice ($n = 41$, $n_{α1 \ KO} = 21/14$ trained and 7 untrained/and $n_{floxed \ control} = 20/13$ trained + 7 untrained/; male; aged 10–12 weeks) were used for the study. C57BL/6J mice were obtained from JANVIER SAS laboratory (France). α1 KO mice were generated as it is described by Vicini et al. (2001). All mice were bred and maintained in polycarbonate cages Type II (207 mm × 140 mm × 265 mm, Ehret, Austria) filled with autoclaved wood chips (Ligncell select, Rettenmaier, Austria) in the core unit of Biomedical Research, Division of Laboratory Animal Science and Genetics, Medical University of Vienna. The animals were housed in groups with access to autoclaved Altromin standard rodent diet (Altromin, Germany) and water ad libitum, and maintained under standard conditions (ambient temperature of 22 ± 1 °C, relative humidity of 50 ± 10 %, light/dark cycle of 14:10, and ventilation with 100 % fresh air that resulted in an air change rate of 15 times per hour). The room was illuminated with artificial light at an intensity of about 200 lx in 2 m from 5 a.m. to 7 p.m. The experiments were carried out under a license obtained from the Federal Ministry of Education, Science and Culture which includes an ethical evaluation of the project (Project: BMWF-66.009/0240-II/10b/2009). Housing and maintenance of animals were in compliance with European and national regulations (Ghafari et al. 2012).

Genotyping of GABA$_A$R α1 KO mice

Genomic DNA from mouse tails was isolated by DNeasy Blood & Tissue Kit from QIAGEN. The mouse line was genotyped by polymerase chain reaction (PCR) of genomic DNA using two different primer combinations. The floxed α1 allele (α1+) that was functionally equivalent to the wild type allele was amplified using the primers KO-α1-F

(5′-CAAGTTGTTACTTACCTTTCTGCTTCC-3′, sense) and α1-1502-R (5′-GAACCACGCTTTTGCCATCC-3′, antisense, exon 8) that resulted in a 750 bp band. The α1 knockout allele (α1−) containing a cre-mediated global deletion of exon 8 of the α1 gene was amplified using the primers KO-α1-F (5′-CAAGTTGTTACTTACCTT TCTG CTTCC-3′, sense) and KO-α1-R (5′-CAAGTATTCAGGC TGCTTTGCAG-3′, antisense), and resulted in a 600 bp band. Heterozygous α1−/+ breeding pairs were crossed to give homozygous mutant and wild type littermate controls for the experiments. PCR reactions were performed with Illustra PuReTaq Ready-To-Go PCR beads (GE Health-care, Little Chalfont, Buckinghamshire, UK) under the following conditions: 1 min 94 °C and 35 cycle (15 s 94 °C, 15 s 54 °C, 45 s 72 °C) for the primer pair of KO-α1-F + KO-α1-R, and 1 min 94 °C 32 cycle (15 s 94 °C, 15 s 58 °C, 45 s 72 °C) for the primer pair KO-α1-F + α1-1502-R.

Behavioral studies: multiple T-maze (MTM)

The MTM is one of the spatial learning tasks in which animals learn to find the goal box based on their memory of previously visited arms and was carried out as described elsewhere (Patil et al. 2009) with minor modifications. The MTM was constructed of wood and consisted of a platform with seven choice points, dimensions of 150 cm × 130 cm × 15 cm and a path width of 8 cm (Fig. 1a). Before testing, mice were deprived of food for 16 h to motivate food searching. Mice were then placed in a start box (diameter: 10.5 cm) and allowed to search the reward. The trial was considered to be completed when mice either reached the goal box or failed to find it within 5 min. In the goal box mice were allowed to consume a small piece of a food pellet provided as a reward. Immediately after each trial, the entire maze was cleaned with 1 % incidin® solution (Incidin extraN, Lohmann and Rauscher, Austria) to remove possible cues. Ten mice were used as untrained controls (yoked controls) that spent the same time in the MTM without learning performance as there was no reward in the goal box. After testing, animals were returned to their cage, given food as to preserve their body weight (120 g/kg) and were kept starving for the following day's task. Mice were trained for four days, with three trials per day having 20 min interval between each trial. Trials were recorded using computerized tracking/image analyzer system (video camcorder: 1/3 in. SSAMHR EX VIEW HAD coupled to the computational tracking system: TiBe-Split). The system provides the following parameters: correct or wrong decision, path length, speed and latency to reach the goal box. Six hours following the last training on day 4, animals were euthanized by neck dislocation. Hippocampi were taken from the brain within 1 min and kept at −80 °C until analyses.
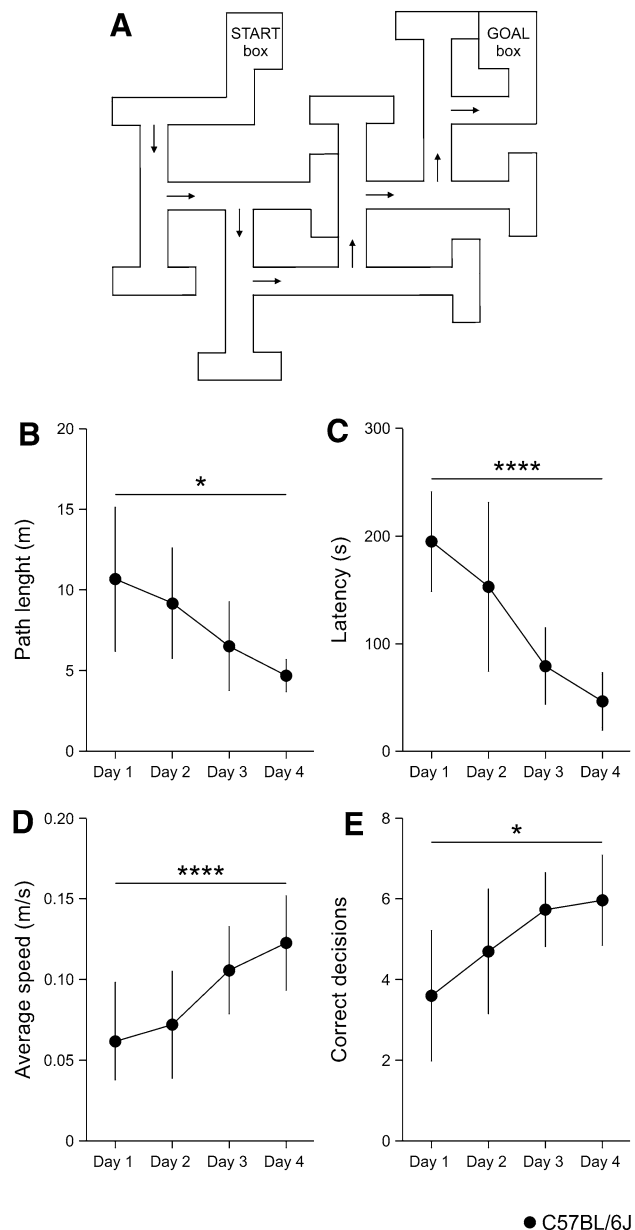

Fig. 1 Training pattern of C57BL/6J mice in the multiple T-maze (MTM). **a** Schematic illustrates the MTM. **b–e** Statistical analysis of parameters measured across the training showing that C57BL/6J mice learned the task: **b** path length (m), **c** latency (s), **d** average speed (m/s) and **e** correct decisions. Data are represented as mean ± SD and were analyzed by repeated measures one-way ANOVA with subsequent Bonferroni's multiple comparisons test ($n = 10$; *$p < 0.05$ and ****$p < 0.0001$; *asterisks* linked to lines refer to post hoc comparisons of day 4 to day 1)

Sample preparation

Enrichment of plasma membrane fraction

Hippocampi of mice were homogenized in ice-cold homogenization buffer [10 mM HEPES, pH 7.5, 300 mM

sucrose, one complete protease inhibitor tablet (Roche Molecular Biochemicals, Mannheim, Germany) per 50 ml] on Ultra-Turrax® (IKA, Staufen, Germany). The homogenate was centrifuged at $1000 \times g$ for 10 min at 4 °C and the pellet was discarded. The supernatant was centrifuged at $50,000 \times g$ for 30 min at 4 °C in an ultracentrifuge (Beckman Coulter Optima® L-90 K). Subsequently, the pellet was resuspended in washing buffer (homogenization buffer without sucrose), kept on ice for 30 min and centrifuged at $50,000 \times g$ for 30 min at 4 °C. Crude membrane pellets were further purified to enrich plasma membrane fractions using sucrose gradient ultracentrifugation as described previously (Ghafari et al. 2012). Briefly, sucrose density gradients were formed by carefully layering 69, 54, 45, 41, and 37 % (w/v) sucrose solutions on top of each other. Membrane pellets were resuspended in homogenization buffer and were layered on top of the sucrose gradient. Samples were centrifuged at $70,000 \times g$ for 3 h at 4 °C. After centrifugation, the 41 % fraction was collected from the sucrose interface, diluted ten times with the homogenization buffer and spun down at $100,000 \times g$ for 30 min at 4 °C. After discarding the supernatant, the pellet was stored at −80 °C until use.

Extraction of native protein complexes

Membrane pellets obtained from the 41 % fraction of sucrose gradient ultracentrifugation were solubilized in extraction buffer (1.5 M 6-aminocaproic acid, 300 mM Bis–Tris, pH 7.0) containing Triton X-100 at 2 % (v/v) final concentration. Samples were incubated on ice for 1 h with vortexing in every 10 min. Following solubilization, samples were cleared by centrifugation at $20,000 \times g$ for 60 min at 4 °C. The protein content was estimated using BCA protein assay kit (Pierce, Rockford, IL, USA).

Blue native: polyacrylamide gel electrophoresis (BN-PAGE)

Membrane protein extracts were mixed with BN-PAGE loading buffer [5 % (w/v) Coomassie G250 in 750 mM 6-aminocaproic acid] in a ratio of 100–16 μl, respectively, and 50 μg of samples were loaded onto gels. BN-PAGE was performed in a PROTEAN II xi Cell (BioRad, Germany) using 4 % stacking and 5–18 % separating gels. The BN-PAGE gel buffer contained 500 mM 6-aminocaproic acid and 50 mM Bis–Tris (pH 7.0), the cathode buffer contained 50 mM Tricine, 15 mM Bis–Tris (pH 7.0) and 0.05 % (w/v) Coomassie G250 and the anode buffer contained 50 mM Bis–Tris (pH 7.0). The voltage was set to 50 V for 1 h then to 75 V for 6 h and finally, it was sequentially increased to 400 V (maximum current 15 mA/gel, maximum voltage 500 V) until the dye front reached

the bottom of the gel (Ghafari et al. 2012). Native high molecular mass markers (Invitrogen, Carlsbad, CA, USA) were used to estimate the size of GABA$_A$R complexes.

Two-dimensional (2D) gel electrophoresis: BN/SDS-PAGE

For detection of the GABA$_A$R α1 subunit in individual complexes, vertical sample lanes from BN-PAGE were excised and equilibrated for 30 min in equilibration buffer [1 % (w/v) SDS and 1 % (v/v) 2-mercaptoethanol] with gentle agitation to bring previously separated native protein complexes into denatured and reduced conditions. Following a brief wash with MilliQ water, gel lanes were rinsed twice with SDS-PAGE electrophoresis buffer [25 mM Tris–HCl, 192 mM glycine and 0.1 % (w/v) SDS, pH 8.3] and placed onto SDS-PAGE gels. SDS-PAGE was performed in a PROTEAN II xi Cell using 4 % stacking and a 5-15 % separating gels. Electrophoresis was carried out at 12 °C with an initial current of 50 V (during the first hour). Voltage was then increased to 100 V for the next 12 h (overnight) and increased to 150 V until the dye front reached the bottom of the gel (Ghafari et al. 2012).

Western blot (WB)

Membrane proteins were transferred from BN-PAGE and BN/SDS-PAGE gels to PVDF membranes. After blocking for 1 h with 10 % (w/v) non-fat dry milk in 0.1 % (v/v) TBST [100 mM Tris–HCl, 150 mM NaCl, pH 7.5, 0.1 % (v/v) Tween 20], membranes were incubated overnight at 4 °C with diluted primary antibodies: rabbit anti-mouse GABA$_A$R α1 (29/18, 17.9.2002, 346 μg/ml, 1:3000), rabbit anti-mouse GABA$_A$R α5 (30/4, 25.4.2004, 385 μg/ml, 1:2500) and rabbit anti-mouse GABA$_A$R γ2 (21/1, 21.6.1999, 326 μg/ml, 1:3000). Primary antibodies were detected using horseradish peroxidase-conjugated anti-rabbit IgG (1:10,000, Abcam, Cambridge, UK) and membranes were developed with ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK). Optical densities (OD) of immunoreactive bands were measured using ImageJ software (NIH, http://rsb.info.nih.gov/ij/). Loading controls for the BN-PAGE/WB were carried out according to Welinder and Ekblad (2011). For correlation analysis of immunoreactivity referring to individual α1, α5 and γ2 subunit-containing complexes, Pearson's correlation coefficients were calculated and analyzed similarly as written below in the study of colocalization.

Immunoprecipitation (IP)

Total hippocampal membrane fractions were suspended in lysis buffer containing 1 % (v/v) Triton X-100, 150 mM

NaCl, 1 mM EDTA, 50 mM Tris–HCl (pH 8.0), 10 mM NaF, 10 mM Na_3VO_4 and protease inhibitor cocktail (Roche, Mannheim, Germany) with incubation on a rotating shaker for 1 h at 4 °C. After centrifugation at $15,300 \times g$ for 10 min at 4 °C, supernatants were incubated with affinity purified rabbit antibody against $GABA_AR$ $\alpha 1$ subunit (Anti-$GABA_AR$ $\alpha 1$ Antibody, Millipore) overnight at 4 °C and subsequently with protein G agarose beads (GE Healthcare, Uppsala, Sweden) for 4 h at 4 °C with gentle rotation. After washing five times with the same lysis buffer, bound proteins were eluted with sample buffer containing 125 mM Tris–HCl (pH 6.8), 4 % (w/v) SDS, 20 % (v/v) glycerol, 10 % (v/v) 2-mercaptoethanol, and 0.02 % (w/v) bromophenol blue at 95 °C for 3 min. Samples were then loaded onto 10 % SDS–polyacrylamide gels, electrophoresed and subsequently processed for LC–MS/MS analysis as described in other subsections.

In-gel digestion and LC–MS/MS analysis

Spots corresponding to the immunoreactivity given by the antibody against $GABA_AR$ $\alpha 1$ subunit following BN/SDS-PAGE and bands derived from SDS-PAGE following IP were picked from the gel and processed as follows. Gel pieces were initially washed with 50 mM ammonium bicarbonate and then two times with washing buffer [50 mM ammonium bicarbonate and 50 % (v/v) acetonitrile], each time for 30 min with vortexing. Afterwards, gel pieces were dehydrated with 100 % acetonitrile for 10 min and dried using a SpeedVac concentrator (Eppendorf, Germany). Reduction of cysteine residues was carried out with 10 mM dithiothreitol (DTT) solution in 100 mM ammonium bicarbonate (pH 8.6) for 60 min at 56 °C. Afterwards, gel pieces were incubated in 55 mM iodoacetamide (IAA) solution in 100 mM ammonium bicarbonate buffer (pH 8.6) for 45 min at 25 °C in dark to achieve alkylation of cysteine residues. Following two washes in washing buffer for 15 min with vortexing, gel pieces were dehydrated in 100 % acetonitrile and dried using SpeedVac. Dried gel pieces were reswollen and incubated in trypsin (Promega, Germany) or in chymotrypsin (Roche, Germany) solutions at the concentration of 12.5 ng/µl in 25 mM ammonium bicarbonate overnight at 37 °C or at 25 °C, respectively. Thereafter, supernatants were collected, and peptides were extracted with 50 µl of 0.5 % (v/v) formic acid/20 % (v/v) acetonitrile two times for 20 min in a sonication bath. Peptide extracts were pooled and concentrated to approximately 20 µl in SpeedVac. Final volumes were adjusted with HPLC grade water (Sigma, Germany) prior injection and samples were subsequently analyzed by nano-LC-ESI-(CID/ETD)-MS/MS (Ultimate 3000 nanoRSLC System, Thermo Fisher Scientific, Sunnyvale, CA, USA and HCTultra PTM Discovery System,

Bruker, Germany) according to the protocol published by Kang et al. (2009). Detailed MS data are available on request.

Immunohistochemistry (IHC) and confocal laser scanning microscopy

Colocalization of $GABA_AR$ $\alpha 1$ and $\alpha 5$ subunits in CA1 and dentate gyrus of dorsal hippocampus was analyzed on coronal sections from perfusion-fixed brains processed for double-immunofluorescence staining (Fig. S2). Briefly, untrained and trained C57BL/6J mice ($n = 5$–5) were anesthetized and perfused transcardially, first with saline and then with 4 % paraformaldehyde in 0.1 M sodium phosphate buffer (PBS, pH 7.4). After perfusion, the brains were taken out and post-fixed overnight at 4 °C followed by cryoprotection with 30 % (w/v) sucrose in 0.1 M PBS (pH 7.3). Brains were cut into 80 µm thick sections with a cryostat (LEICA CM3050S) and immunohistochemistry was performed on three sections of the hippocampal region from each animal.

Tissues were processed by free-floating as follows: sections were incubated in blocking solution [0.1 M PBS, 0.3 % (v/v) Triton X-100, 10 % (v/v) normal donkey serum] for 1 h at 23 °C then with rabbit anti-mouse $GABA_AR$ $\alpha 1$ antibody (Alomone labs., Jerusalem, Israel, 1:100) and goat anti-mouse $GABA_AR$ $\alpha 5$ antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:100) for 72 h at 4 °C on a shaker. Sections were then rinsed with 0.1 M PBS (pH 7.4) and incubated with secondary antibodies (anti-goat IgG labeled with Alexa Fluor 647, 1:1000, Molecular Probes, Invitrogen Corporation, Carlsbad, CA and anti-rabbit IgG Alexa Fluor 555, 1:1000, Cell Signaling Technology, Boston, MA, USA) for 1 h at 23 °C. After rinsing again with 0.1 M PBS (pH 7.3), sections were dried and cover-slipped with fluorescence mounting medium (DAKO, Glostrup, Denmark). Sections were then examined on a Zeiss LSM 700 confocal laser-scanning microscope (LSM 700, Carl Zeiss GmbH, Jena, Germany) using $20\times$ to $63\times$ oil immersion objectives (1.4NA) with the pinhole set at 1 Airy unit. 8-bit images were acquired from each slice multiple times in regions of pyramidal cell layer (SP) of CA1 and the granular cell layer (GCL) of dentate gyrus using LSM 700 software (Carl Zeiss GmbH, Jena, Germany) at 1024×1024 pixel resolution. Contrast and sharpness of the images were adjusted by using the levels and sharpness commands in Adobe Photoshop CS 5 (Adobe Systems, San Jose, CA, USA). Specificity of immunostaining was confirmed by control experiments with omission of primary antibody (data not shown).

Images were analyzed with ImageJ software and colocalization was measured on single confocal sections using

the plugin 'Colocalization Finder' in ImageJ. Measured data are given by the Pearson's correlation coefficient which describes the correlation of intensity distributions between channels. Its values range between −1, 0 and 1, where 0 indicates no significant correlation and 1 indicates complete positive correlation (Heja et al. 2012; David et al. 2007).

Statistical analysis

Quantified data are shown as mean ± SD. Measured parameters in the MTM were daily averaged for each animal and analyzed by repeated measures (RM) one- or two-way ANOVA with subsequent Bonferroni's multiple comparisons test. Data obtained from WB and IHC were analyzed by unpaired two-tailed t test. Statistical analysis was performed using GraphPad Prism 6 (San Diego, CA, USA). For detailed statistics see Table S1. Figures were created using CorelDRAW® Graphics Suite X7 (Ottawa, Canada) graphic design software.

Results

Levels of α1- and α5-containing hippocampal GABA$_A$R complexes are elevated following spatial learning in the MTM

C57BL/6J mice were trained in the MTM (Fig. 1a) and quickly learned the task. Improved performance is demonstrated by a significant decrease in path length (Fig. 1b) and latency (Fig. 1c) to reach the goal box as well as by a gradual increase in average speed (Fig. 1d) and number of correct decisions (Fig. 1e) during the training. Following the behavioral task, animals were taken for biochemical analysis (see below). Another set of C57BL/6J mice was also trained in the MTM and used for IHC studies. They performed in a way similar to that is presented in Fig. 1 (data not shown).

Training effect on the level of α1, α5, and γ2 subunit-containing GABA$_A$R complexes in hippocampi of C57BL/6J mice was investigated by BN-PAGE/WB analysis. According to high molecular weight markers GABA$_A$Rs were detected in the BN gel between 480 and 720 kDa giving estimation about the size of the entire receptor machinery. Densitometry analysis of immunoreactivity revealed that spatial learning in the MTM induces a significant increase in the level of α1 (Fig. 2a) and α5 (Fig. 2b) subunit-containing GABA$_A$R complexes. Furthermore, the amount of GABA$_A$Rs containing the γ2 subunit was also elevated in trained C57BL/6J mice compared to untrained controls (Fig. 2c) but it was at the borderline of statistical significance (Table S1). These data

demonstrate that levels of α1- and α5-containing hippocampal GABA$_A$R complexes are elevated by spatial learning and memory formation (for detailed statistics see Table S1).

Spatial learning induces increased colocalization of hippocampal GABA$_A$R α1 and α5 subunits

To test whether training-induced elevated levels of α1 and α5 subunit-containing hippocampal GABA$_A$Rs can lead to the assembly of complexes comprising both, α1 and α5 subunits, the degree of their colocalization was investigated. Hippocampal sections from untrained and trained animals were immunostained for both subunits as represented in Fig. S2. Multiple images from each section were taken and colocalization of α1 and α5 subunits was measured in the pyramidal cell layer of CA1 and the granule cell layer of dentate gyrus regions (Fig. 3). Measured data are given by the Pearson's correlation coefficient which describes the correlation of intensity distributions between channels. Its values range between −1, 0 and 1, where 0 indicates no significant correlation and 1 indicates complete positive correlation (Heja et al. 2012; David et al. 2007). Some degree of colocalization was observed in both, untrained and trained C57BL/6J mice (Fig. 3e). However, its level was significantly higher in trained animals (Fig. 3c–e) compared to the untrained group (Fig. 3a, b, e) in both subregions suggesting training-induced formation of GABA$_A$R complexes containing both, α1 and α5 subunits (for detailed statistics see Table S1).

Existence of α1, α5 and γ2 subunits in the same high molecular weight hippocampal GABA$_A$R complex

To further investigate the composition of GABA$_A$R complexes, additional biochemical experiments were performed as follows. First of all, the presence of the α1 subunit in high molecular weight receptor complexes was confirmed by 2D BN/SDS-PAGE coupled to WB and LC–MS/MS analysis (Fig. 4a, b, d). As indicated by the arrow in Fig. 4b, immunoreactivity against the α1 subunit in the second dimension at the expected molecular weight (∼50 kDa) was observed along the whole length of BN gel strip which implies that high molecular weight GABA$_A$R complexes comprising the α1 subunit. This was further verified by the identification of α1 subunit via LC–MS/MS analysis following in-gel digestion by either trypsin or chymotrypsin (Fig. 4d) of spots (displayed by the arrow on Fig. 4a) corresponding to the WB signal. Afterwards, IP of the α1 subunit was carried out and its coeluted proteins were separated by SDS-PAGE (Fig. 4c). As Fig. 4c shows, bands were picked from the gel, from which α1 (both rectangles), α5 and γ2 subunits (lower rectangle) were
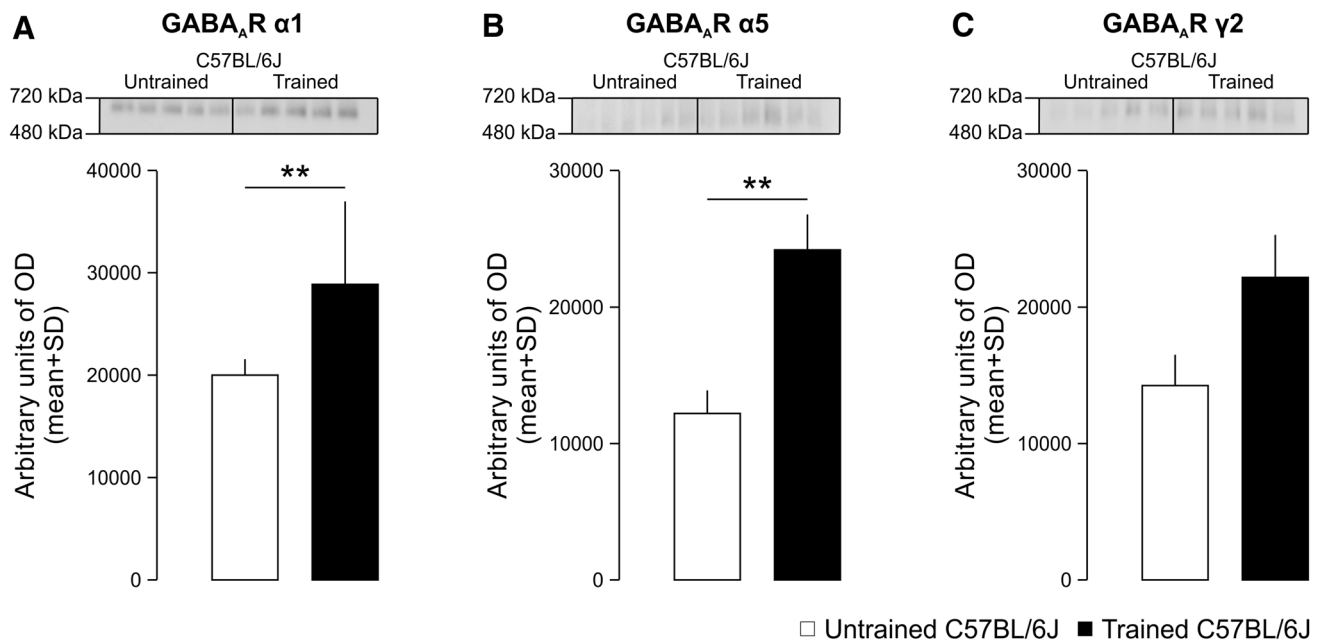
Fig. 2 Training-induced changes in levels of hippocampal GABA$_A$R complexes in C57BL/6J mice. Representative images and quantification of BN-PAGE/WB analysis indicate training-induced significant increase in the amount of **a** α1, **b** α5 but not **c** γ2 subunit-containing complexes (detected between 480 and 720 kDa). Optical densities (OD) of immunoreactive bands were measured and are shown as mean ± SD. Data were analyzed by unpaired two-tailed t test. ($n = 10$–10; **$p < 0.01$)
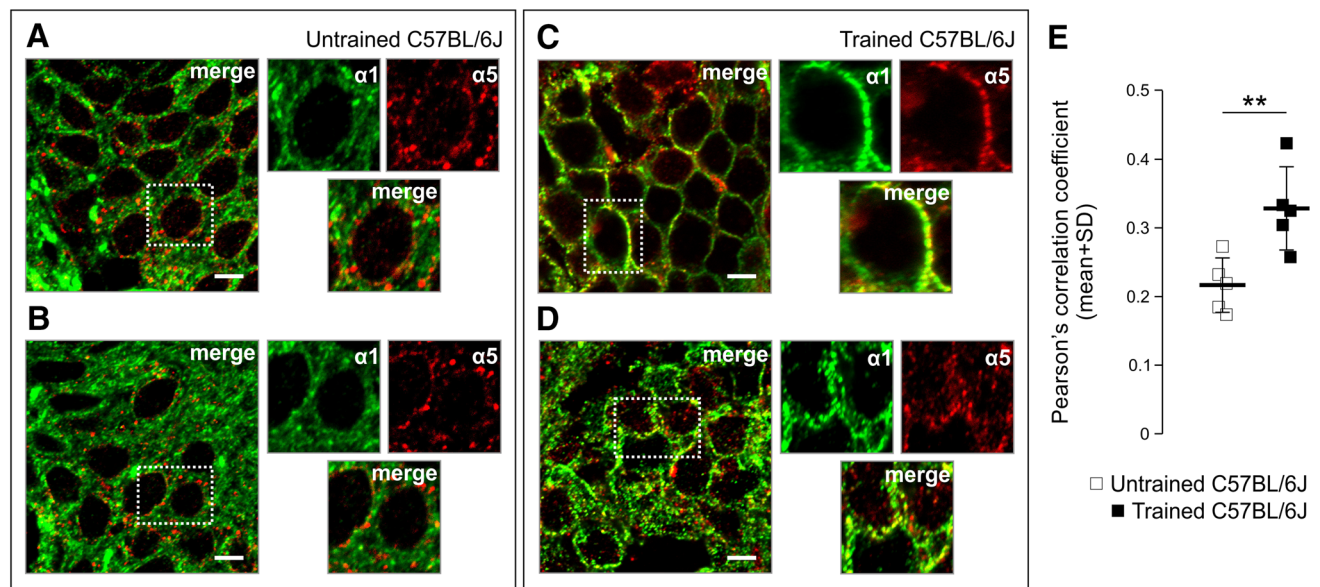

Fig. 3 Training-induced increase in colocalization of GABA$_A$R α1 and α5 subunits in CA1 and dentate gyrus regions of C57BL/6J mice. **a–d** Representative images of α1 (*green*) and α5 (*red*) stainings in the dentate gyrus showing two independent experiments from both, **a**, **b** untrained and **c**, **d** trained groups. Colocalization is indicated by yellow color formation. **e** The level of colocalization was quantified by the Pearson's correlation coefficient (shown as mean ± SD) and analyzed by unpaired two-tailed t test ($n = 5$–5; **$p < 0.01$). *Scale bars 5 µm*

identified by LC–MS/MS analysis (Fig. 4d). The presence of α1 subunit in the upper band at about 100–150 kDa is probably due to aggregation. Coprecipitation of α5 and γ2 subunits with the α1 subunit suggests their possible existence in the same GABA$_A$R complex. In addition, correlation analysis of WB signal densities (Fig. 2) referring to α1, α5 and γ2 subunit-containing complexes was performed further assessing the possible interaction
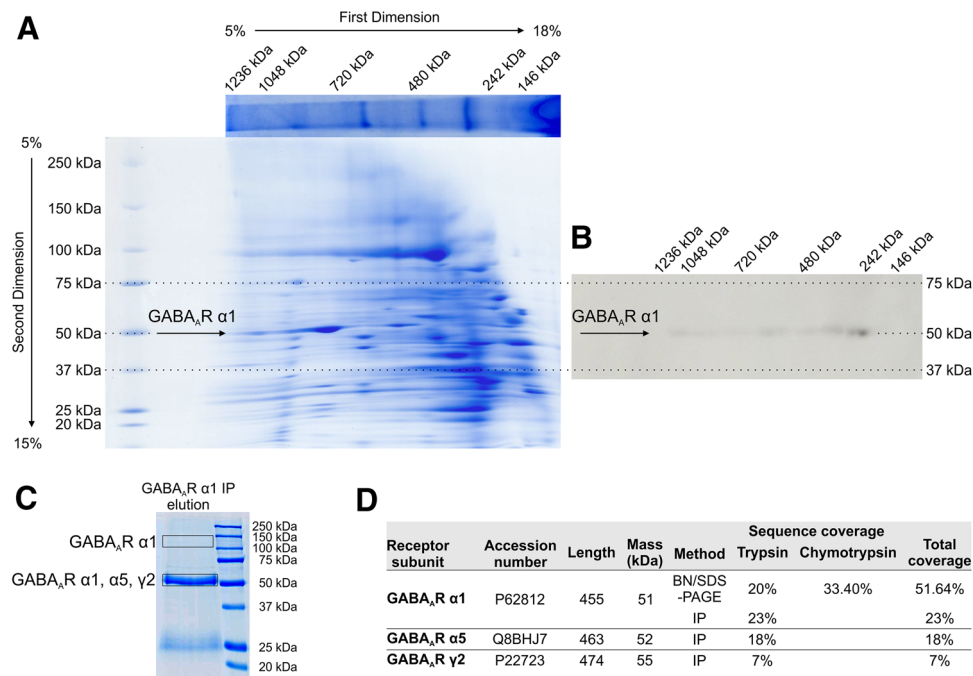
Fig. 4 Formation of hippocampal GABA$_A$R complexes containing α1, α5 and γ2 subunits. **a, b** Representative images of two-dimensional (2D) BN/SDS-PAGE/WB analysis reveal the presence of the αl subunit in high molecular weight GABA$_A$R complexes. Spots (*arrow* on **a**) corresponding to immunoreactivity against the αl subunit (*arrow* on **b**) were picked for MS analysis. **c** Representative image of SDS-PAGE following IP of the αl subunit. α5 and γ2 subunits were coeluted with the αl subunit as they were identified using MS (**d**) from the bands indicated by *rectangles*. **d** Data show identification of α1, α5 and γ2 subunits in α1 subunit-containing complexes by MS analysis of samples derived from 2D BN/SDS-PAGE and IP/SDS-PAGE. (Sequence coverage is shown following either trypsin or chymotrypsin digestion as well as in total.)

between these subunits. This analysis was used to link levels of individual GABA$_A$R complexes which might predict possible assembling patterns as they are modulated in strong correlation. Significant positive correlations were observed between α1 and α5 ($R = 0.546$; $p = 0.016$) as well as α1 and γ2 ($R = 0.460$; $p = 0.048$) subunit-containing GABA$_A$Rs complexes, however, no correlation was detected between α5 and γ2 subunit-containing complexes. Taken together, these results pinpoint the possibility that α1, α5 and γ2 subunits exist in the same GABA$_A$R.

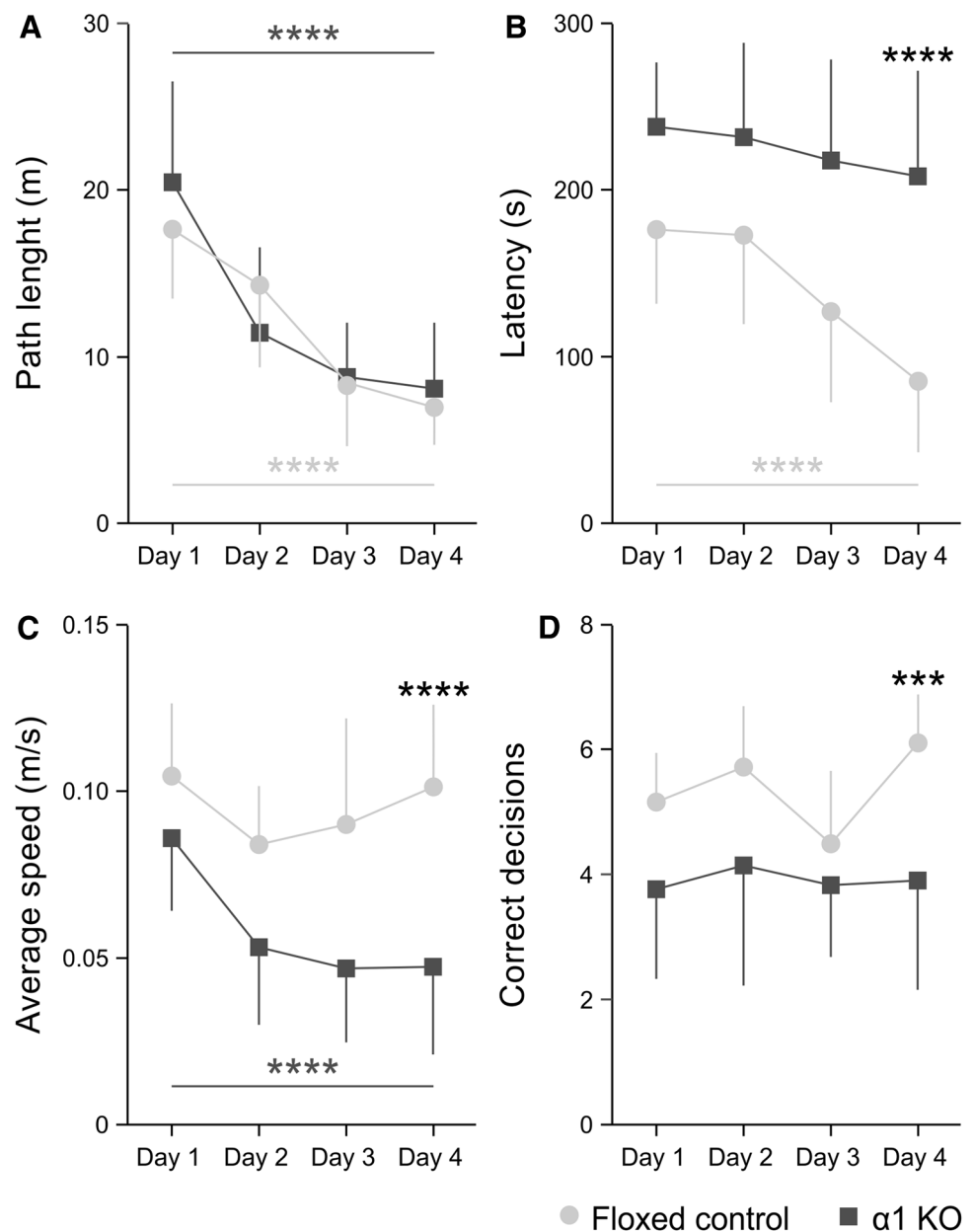## Spatial learning in the MTM is impaired in mice lacking the GABA$_A$R α1 subunit

To determine the effect of α1GABA$_A$R complexes on spatial learning and memory formation, α1 KO mice and their floxed controls were also tested in the MTM (Fig. 5). α1 KO mice reached the goal box within 5 min (Fig. 5b) and showed a decreasing tendency in their average speed (Fig. 5c). Their path length was comparable to that of the floxed controls (Fig. 5a). However, key parameters of the MTM indicated impaired memory formation: at the end of the task, the latency to reach the goal box was significantly longer (Fig. 5b) and the number of correct decisions was significantly lower (Fig. 5d) of α1 KO mice in contrast to the controls. These data indicate

that while the performance of floxed controls was similar to that of C57BL/6J mice (Fig. 1), α1 KO animals showed impaired spatial learning in the MTM.

Next, to see how α1 deficiency affects hippocampal GABA$_A$R levels, α1, α5 and γ2 subunit-containing complexes were compared between α1 KO and floxed control mice (Fig. 6). As expected, there was no α1 immunoreactivity in α1 KO mice (Figs. 6a, 7a). The level of α5 subunit-containing complexes was not affected by knocking out the α1 subunit (Fig. 6b) while the amount of γ2 subunit-containing GABA$_A$Rs (γ2GABA$_A$Rs) was significantly reduced (Fig. 6c). Levels of these receptor complexes were also investigated following training in the MTM to reveal their relation to impaired memory formation (Fig. 7). The level of α5GABA$_A$Rs was significantly elevated in trained α1 KO mice compared to their trained controls (Fig. 7b). In turn, the level of γ2GABA$_A$Rs was remarkably lower in trained α1 KO mice, similarly to that observed between untrained KO and wild type animals (Fig. 7c). In conclusion, α1 deficiency led to decreased levels of γ2GABA$_A$Rs and caused impaired performance in a spatial learning task. Impaired memory formation was correlating with increased levels of α5GABA$_A$R complexes in α1 KO mice in comparison to floxed controls (for detailed statistics see Table S1).

Fig. 5 Training pattern of GABA$_A$R α1 KO and floxed control mice in the multiple T-maze. Statistical analysis of parameters measured across the training showing impaired performance of α1 KO mice in the task: **a** path length (m), **b** latency (s), **c** average speed (m/s) and **d** correct decisions. Data are represented as mean ± SD and were analyzed by repeated measures two-way ANOVA with subsequent Bonferroni's multiple comparisons test [$n_{control} = 13$, $n_{KO} = 14$; ***$p < 0.001$ and ****$p < 0.0001$; *asterisks* linked to lines refer to post hoc comparison of day 4 to day 1 (*light gray* for floxed controls and *dark gray* for α1 KO mice); *black asterisks* alone refer to post hoc comparison of the two genotypes on day 4]

Discussion

The major outcome of the current study is that spatial learning in the MTM is paralleled by increased levels of GABA$_A$R complexes containing α1 and α5 subunits in the hippocampus of C57BL/6J mice (Fig. 2a, b). Besides, levels of receptor complexes containing the γ2 subunit showed a strong tendency to increase (Fig. 2c) but, due to higher abundance of the γ2 subunit as compared to the other two subunits and a slightly larger variability of data, this increase was at the borderline of statistical significance (Table S1). Accordingly, spatial learning is impaired by α1 deficiency (Fig. 5).

GABA$_A$ receptors containing two different alpha subunits seem to exist with high probability (Olsen and Sieghart 2008). In addition, evidence has been provided that α1 and α5 subunits might be associated with each other in the same receptor subtype (Araujo et al. 1999; Sieghart and Sperk 2002). Thus, antibodies against α5 subunits could coprecipitate α1 subunits and those directed against α1 subunits could coprecipitate α5 subunits. The latter finding was also confirmed in the present study (Fig. 4). In addition, copurification of α5 subunits with multiple α, β, and γ subunits has been proven recently via MS analysis (Ju et al. 2009). The present study extends previous studies by providing mass spectrometric evidence for the presence of

Fig. 6 Levels of hippocampal GABA$_A$R complexes in α1 KO and floxed control mice. Representative images and quantification of BN-PAGE/WB analysis indicate **a** the lack of αl-containing GABA$_A$Rs, **b** comparable level of α5 but **c** significantly lower level of γ2 subunit-containing GABA$_A$R complexes in the αl KO mice compared to the floxed controls (complexes were detected between 480 and 720 kDa). Optical densities (OD) of immunoreactive bands were measured and are shown as mean ± SD. Data were analyzed by unpaired two-tailed *t* test ($n_{α5} = 7$–7, $n_{γ2} = 6$–6; **$p < 0.01$)
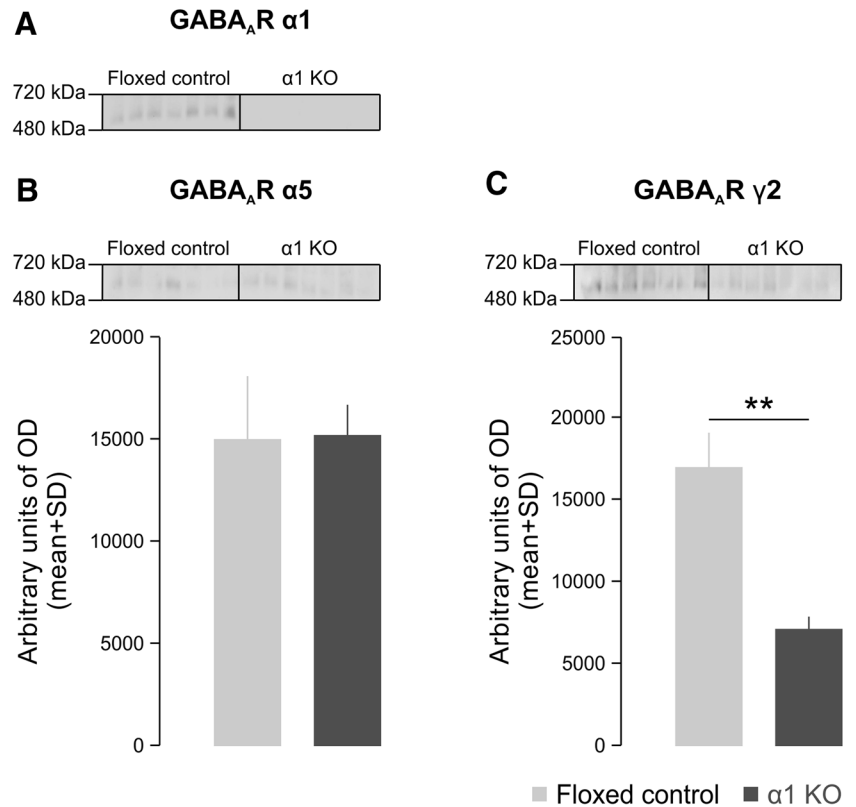

Fig. 7 Levels of hippocampal GABA$_A$R complexes in α1 KO and floxed control mice trained in the multiple T-maze. Representative images and quantification of BN-PAGE/WB analysis indicate **a** the lack of αl-containing GABA$_A$Rs, **b** significantly higher level of α5 and **c** significantly lower level of γ2 subunit-containing GABA$_A$R complexes in trained αl KO mice compared to trained floxed controls (complexes were detected between 480 and 720 kDa). Optical densities (OD) of immunoreactive bands were measured and are shown as mean ± SD. Data were analyzed by unpaired two-tailed *t* test. ($n = 6$–6; **$p < 0.01$ and ***$p < 0.001$)
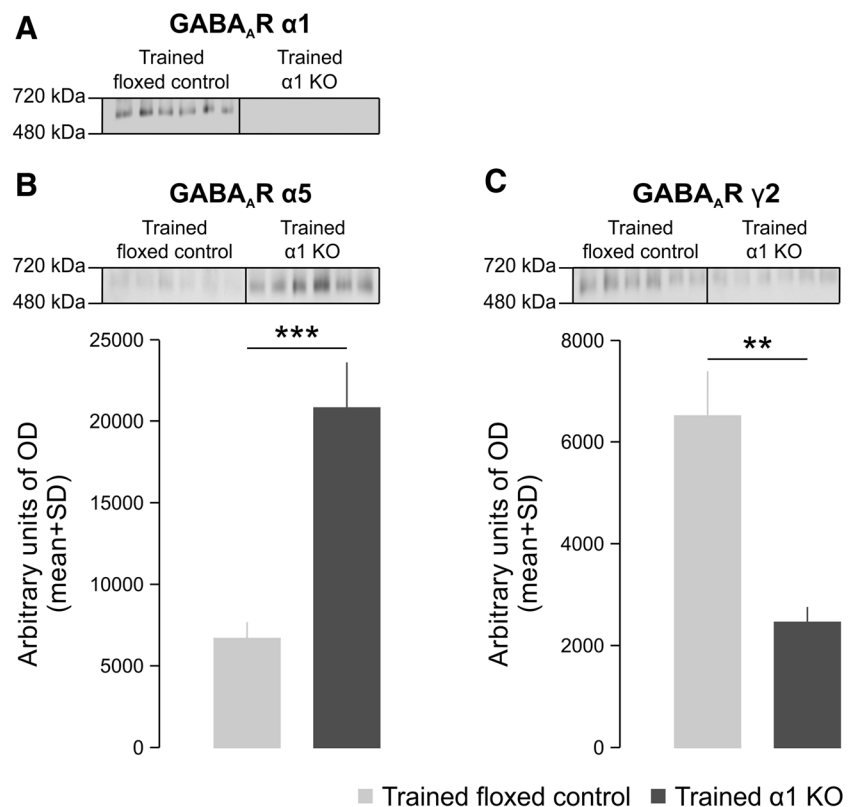
high molecular mass GABA$_A$R complexes containing α1, α5 and γ2 subunits in the hippocampus (Fig. 4). Whether other GABA$_A$R subunits were also present in these high molecular mass complexes was not investigated. The apparent molecular weight of this complex on BN-PAGE/WB was between 480 and 720 kDa which is higher than the expected molecular mass of a GABA$_A$R alone (250 kDa). This may indicate that either additional receptors or regulatory proteins such as protein kinases and protein phosphatases are constituents of this receptor complex.

Coprecipitation of α1 and α5 subunits and concomitant increased levels of their complexes during training in the MTM might have been caused by the coassembly of α1 as well as α5 subunits in the same receptor subtype, possibly forming α1α5βγ2 receptors. Alternatively, it could have been caused by an increased formation of complexes containing a mixture of α1βγ2 and α5βγ2 receptors that are associated with each other. Both possibilities are consistent with and supported by our additional finding that training in the MTM task increased the colocalization of α1 and α5 subunits in the hippocampus as demonstrated by confocal microscopy (Fig. 3). Unfortunately, the methodology applied does not allow us to distinguish between these possibilities.

The involvement of GABA$_A$Rs containing α1 as well as those containing α5 subunits in learning and memory has been demonstrated previously. Targeted disruption of the GABA$_A$R α1 subunit gene caused an impaired performance in the MWM task (Berry et al. 2008) and as demonstrated in the present study, an impaired performance in the MTM task. Targeted disruption of the α5 gene in mice, however, was shown to improve hippocampal-dependent spatial learning in a water maze task (Collinson et al. 2002). Moreover, introduction of a point mutation in the gene of α5 subunit causing the reduction of α5 subunit-containing receptors in the hippocampus facilitated trace fear conditioning (Crestani et al. 2002). Finally, a growing body of evidence highlights cognition enhancing properties of selective inverse agonists (negative allosteric modulators) for α5GABA$_A$Rs in a water maze task (Collinson et al. 2006; Chambers et al. 2004; Dawson et al. 2006). These studies indicate that learning is improved on negative modulation or reduction of α5-containing receptors. On the other hand, a reduction in hippocampal α5GABA$_A$Rs was described to disrupt memory for location of objects in mice (Prut et al. 2010). In addition, the present study indicates that learning in both, wild type (Fig. 2) and α1 KO mice (Fig. 7) is associated with increased levels of GABA$_A$Rs containing the α5 subunit.

α1GABA$_A$Rs are mainly localized synaptically and involved in phasic inhibition of the postsynaptic neuron. α5GABA$_A$Rs have been identified at synaptic as well as at extrasynaptic sites. At synapses, they often colocalize with α1, β2/3, and γ2 subunits, as well as with gephyrin clusters (Christie and de Blas 2002). Extrasynaptically, they are not colocalized with gephyrin but form clusters with radixin (Loebrich et al. 2006) that is essential for α5 subunit anchoring at the actin cytoskeleton. Due to their specific extrasynaptic localization on dendritic spines of pyramidal neurons, they provide a tonic inhibitory control of the excitatory input. Extrasynaptic receptors containing α5 subunits may thus be involved in modulation of dendritic excitability and efficacy of excitatory inputs (Yee et al. 2004). Indeed, how extrasynaptically localized α5GABA$_A$Rs modulate synaptic plasticity thus, what is the impact of tonic inhibition on memory processes, has not yet been elucidated and there is only a limited body of work available assessing these questions (Martin et al. 2010; Bonin et al. 2007).

The discrepancy between the present results indicating that learning is associated with an increase in α5 subunit-containing complexes and previous results indicating that learning is associated with a reduction of α5GABA$_A$R activity, might be explained by learning-induced formation of α1α5βγ2 receptors. Receptors containing two different alpha subunits exhibit properties that are distinct from those containing only a single type of alpha subunits. Moreover, GABA-sensitivity of these receptors depends on the position of the different subunits within the receptor (Minier and Sigel 2004). A learning-induced change in the subunit composition of GABA$_A$Rs might directly influence inhibitory control and learning abilities, however, the properties of α1α5βγ2 GABA$_A$Rs have not yet been investigated in comparison to α5βγ2 receptors. Alternatively, the incorporation of an α1 subunit into α5βγ2 receptors might allow these extrasynaptic receptors to move to synapses and being trapped there by interaction with gephyrin. Lateral movement of α5-subunit-containing receptors from extrasynaptic to synaptic sites has recently been suggested to be essential for learning and memory formation (Hausrat et al. 2015). Such lateral movement could reduce the tonic inhibition of pyramidal cells effecting learning and memory formation. A similar scenario, however, can also be constructed for the possibility that learning is facilitated by an increased complex formation between α1βγ2 and α5βγ2 receptors. Recently, evidence has accumulated that GABA$_A$Rs are able to associate and cross-talk with a variety of other receptors (Shrivastava et al. 2011a). This cross-talk often causes reduced efficacy of GABA for activating GABA$_A$Rs and thus, reduced inhibition of the post-synaptic neuron. It is, therefore, possible that during learning, α1βγ2 receptors might associate with extrasynaptic α5βγ2 receptors and by that might influence GABAergic inhibition and memory formation. Such a mechanism also could be associated with

increased lateral mobility of the associated receptors and a specific synaptic targeting (Shrivastava et al. 2011b). As mentioned before, the present work cannot distinguish between the formation of $\alpha1\alpha5\beta\gamma2$ complexes or an increased association of $\alpha1\beta\gamma2$ and $\alpha5\beta\gamma2$ receptors. Indeed, further studies using specific modulators against these distinct GABA$_A$R subtypes or IHC analysis using multiple labeling against $\alpha1$ and $\alpha5$ subunits together with molecular markers for subcellular localization (such as gephyrin or radixin) would be essential to reveal exact molecular mechanisms behind the above mentioned possibilities.

On targeted disruption of the $\alpha1$ subunit gene, $\alpha1\beta\gamma2$ receptors, that comprise the majority of GABA$_A$Rs in the brain, cannot be formed and the assembly intermediates containing β and $\gamma2$ subunits are unstable and become degraded. This degradation can explain the drastic reduction of $\gamma2$GABA$_A$R complexes observed in $\alpha1$ KO mice while the number of $\alpha5$GABA$_A$Rs remains unchanged as shown in Fig. 6. Furthermore, increased level of $\alpha5$GABA$_A$Rs receptors (Fig. 7) and the lack of $\alpha1$GABA$_A$Rs, probably along with the failure to form $\alpha1\alpha5\beta\gamma2$ complexes, might explain impaired spatial learning abilities of $\alpha1$ KO mice observed in the MTM.

In summary, high molecular mass GABA$_A$R complexes containing $\alpha1$, $\alpha5$ and $\gamma2$ subunits exist in the hippocampus of C57/BL6j mice. Training in the MTM increases the level of $\alpha1$- and $\alpha5$-containing GABA$_A$R complexes and induces increased colocalization of $\alpha1$ and $\alpha5$ subunits. $\alpha1$ deficiency leads to impaired performance in the MTM. These results together suggest either training-induced formation of $\alpha1\alpha5\beta\gamma2$ GABA$_A$Rs or enhanced formation of complexes consisting of $\alpha1\beta\gamma2$ and $\alpha5\beta\gamma2$ receptors affecting spatial learning abilities. Our findings contribute to the understanding how $\alpha1$ and $\alpha5$ subunit-containing GABA$_A$ receptor complexes may be involved in mechanisms of spatial learning and memory formation.

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