Published in final edited form as:

Eur J Neurosci. 2016 September ; 44(5): 2258–2271. doi:10.1111/ejn.13329.

Partial inactivation of GABA_A receptors containing the $\alpha 5$ subunit affects the development of adult-born dentate gyrus granule cells

Francine Deprez^{1,2,¥}, Fabia Vogt^{1,¥}, Amalia Floriou-Servou¹, Carlos Lafourcade¹, Uwe Rudolph^{3,4}, Shiva K. Tyagarajan^{1,2}, and Jean-Marc Fritschy^{1,2,*}

¹University of Zurich, Institute of Pharmacology and Toxicology, 8057 Zurich, Switzerland

²Neuroscience Center Zurich, ETH and University of Zurich ³Laboratory of Genetic

Neuropharmacology, McLean Hospital, Belmont, MA, U.S.A ⁴Department of Psychiatry, Harvard Medical School, Boston, MA, USA

Abstract

Alterations of neuronal activity due to changes in GABA_A receptors (GABA_AR) mediating tonic inhibition influence different hippocampal functions. *Gabra5*-null mice and $\alpha 5$ subunit^(H105R) knock-in mice exhibit signs of hippocampal dysfunction, but are capable of improved performance in several learning and memory tasks. Accordingly, alleviating abnormal GABAergic tonic inhibition in the hippocampal formation by selective $\alpha 5$ -GABA_AR modulators represents a possible therapeutic approach for several intellectual deficit disorders. Adult neurogenesis in the dentate gyrus is an important facet of hippocampal plasticity; it is regulated by tonic GABAergic transmission, as shown by deficits in proliferation, migration, and dendritic development of adult-born neurons in *Gabra4*-null mice. Here, we investigated the contribution of $\alpha 5$ -GABA_ARs to granule cell development, using retroviral vectors expressing eGFP for labeling precursor cells in the subgranular zone. Global $\alpha 5$ -GABA_AR knockout ($\alpha 5$ -KO) mice showed no alterations in migration and morphological development of eGFP-positive granule cells. However, upregulation of $\alpha 1$ subunit-immunoreactivity was observed in the hippocampal formation and cerebral cortex. In contrast, partial gene inactivation in $\alpha 5$ -heterozygous ($\alpha 5$ -het) mice, as well as single-cell deletion of *Gabra5* in newborn granule cells from $\alpha 5$ -floxed mice, caused severe alterations of migration and dendrite development. In $\alpha 5$ -het mice, retrovirally-mediated overexpression of Cdk5 resulted in normal migration and dendritic branching, suggesting that Cdk5 cooperates with $\alpha 5$ -GABA_ARs to regulate neuronal development. These results show that minor imbalance of $\alpha 5$ -GABA_AR-mediated transmission may have major consequences for neuronal plasticity; and call for caution upon chronic therapeutic use of negative allosteric modulators acting at these receptors.

Keywords

adult neurogenesis; Cdk5; dendrites; migration; retrovirus; targeted gene deletion

*Corresponding author: University of Zurich, Institute of Pharmacology and Toxicology, Winterthurerstrasse 190, 8057 Zurich, Switzerland; Tel. +41 44 635 5926; fritschy@pharma.uzh.ch.

¥Equal contribution

Introduction

GABA_A receptors (GABA_ARs) containing the $\alpha 5$ subunit are highly expressed during brain development and have a restricted distribution in adult CNS (reviewed in (Fritschy and Panzanelli, 2014)). Representing <10% of total GABA_ARs, they are most abundant in the hippocampus, amygdala, olfactory bulb, brainstem, and spinal cord (Fritschy and Mohler, 1995; Sur et al., 1998; Serwanski et al., 2006). The majority of $\alpha 5$ -GABA_ARs are located extrasynaptically, mediating tonic inhibition (Caraiscos et al., 2004; Prenosil et al., 2006; Glykys et al., 2008; Belelli et al., 2009; Engin et al., 2015). However, they also contribute to several forms of slow phasic inhibition in the hippocampus, suggesting specialized functions in the control of neuronal excitability (Glykys and Mody, 2006; Prenosil et al., 2006; Zarnowska et al., 2009; Vargas-Caballero et al., 2010).

For instance, $\alpha 5$ -mediated tonic inhibition is essential for management of memory interference, with reduced performance, e.g., in reversal learning and pattern separation tasks in mice lacking the $\alpha 5$ subunit in dentate gyrus granule cells (Engin et al., 2015). The analysis of mutant mice carrying a histidine-to-arginine point mutation at residue 105 ($\alpha 5$ H105R), which have a ~30% decrease in $\alpha 5$ -GABA_ARs in the CNS (Crestani et al., 2002), revealed that these mice exhibit several signs of hippocampal dysfunction, including locomotor hyperactivity, reduced pre-pulse inhibition, impaired memory for the location of objects, and are more resistant to extinction of conditioned fear responses (Yee et al., 2004; Hauser et al., 2005; Prut et al., 2010). Paradoxically, however, these mice also exhibited improved performance in several learning and memory tasks, in particular trace fear conditioning (Crestani et al., 2002; Yee et al., 2004). Improved learning and memory performances are also seen in *Gabra5*-null mice (Collinson et al., 2002). Accordingly, negative allosteric modulators acting specifically at $\alpha 5$ -GABA_ARs have cognitive enhancement properties (Dawson et al., 2006) and allow to revert memory deficits observed in some experimental pathological conditions (Wang et al., 2012). Altered $\alpha 5$ -GABA_AR functions have also been reported in models of schizophrenia (Gill and Grace, 2014) and cognitive disability, including Fragile X syndrome (Martin et al., 2014) and Down syndrome (Martínez-Cué et al., 2013), along with symptomatic improvement following treatment with selective allosteric modulators (reviewed in (Rudolph and Möhler, 2014)). Collectively, these observations point to tonic – and slow phasic (Prenosil et al., 2006; Zarnowska et al., 2009) – inhibitory neurotransmission mediated by $\alpha 5$ -GABA_ARs as a key regulator of hippocampal function for learning and memory.

Tonic inhibition in the dentate gyrus (DG) is mediated by GABA_ARs containing $\alpha 4\delta$ - and $\alpha 5$ -subunits (Glykys et al., 2008). It is also well known to modulate adult neurogenesis in rodent CNS, notably by regulating stem and neural precursor cell proliferation (Liu et al., 2005; Song et al., 2012), migration of newborn neurons in the granule cell layer (GCL), and their dendritic maturation (Duveau et al., 2011). Many of these effects involve $\alpha 4$ -GABA_ARs whereas the contribution of $\alpha 5$ -GABA_ARs has not been established. Therefore, the aim of the present study was to determine whether reduced function of $\alpha 5$ -GABA_ARs upon targeted genetic deletion of *Gabra5* affects specific steps of adult neurogenesis in the DG. To this end, we investigated the development of adult-born granule cells (GCs) birth-

dated and labeled by transduction with a retroviral vector encoding eGFP in global $\alpha 5$ -subunit homozygous knockout ($\alpha 5$ -KO) and heterozygous knockout ($\alpha 5$ -het) mice, compared to wild type control littermates. Furthermore, we investigated the consequences of a cell-autonomous *Gabra5* deletion using the cre/lox system to determine the selective role of $\alpha 5$ -GABA_ARs for the maturation of newborn GCs.

Materials and Methods

Animals

All experiments were performed in accordance with the European Community Council Directives of November 24, 1986 (86/609/EEC) and approved by the cantonal veterinary office of Zurich. In the present study, we used 8 to 12 week-old male C57BL/6J (wild type), $\alpha 5$ -knockout ($\alpha 5$ -KO; allele *Gabra2*^{tm2.2Uru}), heterozygous ($\alpha 5$ -het), $\alpha 5$ -floxed ($\alpha 5^{\text{fl/fl}}$; allele *Gabra2*^{tm2.1Uru}) and heterozygous $\alpha 5$ -floxed ($\alpha 5^{\text{fl/+}}$) mice, which were bred in the dedicated animal facility of the Institute of Pharmacology and Toxicology of the University of Zurich. $\alpha 5^{\text{fl/fl}}$ mice were generated using a replacement-type targeting vector containing loxP-flanked exons 4 and 5 of the *Gabra5* gene (for details, (Engin et al., 2015)). $\alpha 5$ -KO mice were obtained by cre-mediated excision of the floxed exons *in vitro*. Both lines were then back-crossed on the C57BL/6J background for >10 generations. Genotype was determined by PCR analysis from ear biopsies.

Viral vectors

Retroviruses encoding enhanced green-fluorescent protein (eGFP), monomeric red fluorescent protein (mRFP), cyclin-dependent kinase 5 (Cdk5)-GFP, Cre-GFP, or Cre-mCherry constructs were produced by transfecting HEK 293T cells with three separate plasmids containing the capsid (CMV-vsvg), viral proteins (CMV-gag/pol) and transgene (CAG-eGFP, CAG-mRFP, CAG-Cdk5-eGFP, CAG-Cre-eGFP, CAG-Cre-mCherry) under the control of the CAG promoter (including a cytomegalovirus CMS enhancer and chicken β -actin promoter). The supernatant containing the virus was concentrated by ultracentrifugation and diluted in phosphate-buffered saline (PBS). The preparations used had a titer of at least 10^8 cfu/mL, as determined by serial titration upon HEK293 cell transfection.

Stereotaxic injections

Adult mice were anesthetized by inhalation with 2.5–3% isoflurane (Baxter) in oxygen and placed on the stereotaxic frame (David Kopf Instruments). The mice received a bilateral injection of retroviruses encoding eGFP or Cdk5-eGFP (1 μ L) or a 1:1 mixture of retroviruses encoding Cre-eGFP and mRFP (1.5 μ L) into the dorsal hippocampus (anteroposterior = –2 mm, mediolateral = ± 1.5 mm, dorsoventral = –2.3 mm, with Bregma as reference), using a nanoliter injector Nanoject II (Drummond Scientific). After the surgery, the animals received an i.p. injection of 1 mg/kg buprenorphine (Temgesic, Essex Chemicals, Lucerne, Switzerland) and were kept on a warm pad to recover from anesthesia, before being returned to their home-cage.

Tissue preparation for immunohistochemistry

Mice were deeply anesthetized with pentobarbital (Nembutal®; 50 mg/kg, i.p.) and perfused transcardially with 15–20 mL ice-cold, oxygenated artificial cerebrospinal fluid (ACSF), pH 7.4, as described previously (Notter et al., 2014). After perfusion, mice were decapitated and brains were immediately removed on ice and fixed by immersion in 4% paraformaldehyde (dissolved in 0.15 M sodium phosphate buffer, pH 7.4) for 90 or 270 min (immunoperoxidase staining). After cryoprotection in 30% sucrose overnight, 50- μ m-thick coronal sections containing the hippocampus were cut from frozen brains with a sliding microtome and collected in PBS.

Immunofluorescence staining

Immunofluorescence staining was performed by incubating sections with primary antibodies against GFP (and where applicable, mRFP) (Table 1) diluted in PBS (pH 7.4) containing 2% normal goat serum and 0.2% Triton X-100 for 48–72 h at 4°C.

Sections were then washed in 3x PBS for 10 min and incubated at room temperature in secondary antibodies together with DAPI for 30 min. Secondary antibodies conjugated to Alexa 488 (Invitrogen) or Cy3 (Jackson ImmunoResearch) were raised in goat. Afterwards, sections were rinsed again 3 times in PBS and mounted on gelatin-coated slides, before coverslipped with a fluorescence mounting medium (Dako).

Immunoperoxidase staining

Immunoperoxidase staining was performed using diaminobenzidine as a chromophore. Brain sections were incubated overnight at 4°C with primary antibodies (Table 1) diluted in Tris buffer (pH 7.4) containing 2% normal goat serum and 0.2% Triton X-100. They were then washed in Tris-Triton, pH 7.4, for 3 x 10 min and incubated for 30 min at room temperature in secondary biotinylated antibody (1:300, Jackson ImmunoResearch). Sections were then washed again in Tris-Triton, incubated in ABC complex solution for 30 min (Vectastain Elite kit; Vector Laboratories) and transferred for 5–15 min in 0.05% diaminobenzidine tetrahydrochloride (Sigma-Aldrich) dissolved in Tris buffer containing 0.002% H₂O₂, pH 7.7. Finally, sections were washed thoroughly, mounted onto gelatin-coated slides, air-dried overnight, dehydrated, and coverslipped with Eukitt (Erne Chemie). To ensure staining consistency across genotypes, animals, and sections for densitometry analysis of staining intensity, all sections incubated with a given primary antibody were handled together, using the same solutions and incubation times.

Image acquisition and analysis

Four series of experiments were performed: 1) injection of retroviruses encoding eGFP in adult wild type, α 5-het, or α 5-KO littermates; the migration and morphology of labeled GCs were analyzed at 7, 14, 28 and 42 days-post-injection (dpi), using images taken by laser-scanning microscopy (LSM700, Carl Zeiss). 2) Injection of a mixture of retroviruses encoding mRFP and Cre-eGFP in adult α 5^{fl/fl} mice; single (mRFP) and double-labeled (mRFP/Cre-eGFP) GCs were analyzed at the same time-points as above. 3) Injection of retroviruses encoding Cdk5-eGFP in wild type and α 5-het mice, analyzed at 28 dpi. 4) Immunoperoxidase staining for the GABA_AR α 1, α 2, α 3, α 4, and α 5 subunits in adult mice

from all three genotypes. For all quantitative analyses, the observer was blinded relative to genotype.

Migration distance—The distance of migration of newborn GCs was measured in images acquired with a 25x oil immersion objective, and displayed in maximal intensity projection mode. Using DAPI to counterstain cell nuclei in the GCL, the orthogonal distance from the cell center to the base of the GCL was measured. For each animal (3–6 mice per group) and time-point, 20–60 cells were analyzed.

Dendrite morphology—To visualize the dendrites of GCs, confocal images were acquired with a 40x (NA, 1.4) oil immersion objective (pixel size, 120 nm). Each cell was imaged across the entire thickness of the section; taking z-stacks with the LSM700 spaced by 0.7 μm . The complexity of the dendritic tree was quantified by Sholl analysis, using concentric circles, spaced at 10 μm intervals, centered on the cell body. The numbers of intersections were calculated using a Sholl Analysis plugin (Anirvan Ghosh Laboratory, University of California, San Diego, La Jolla, CA). Dendritic morphometry (primary dendrite length, total dendrite length, number of terminal branches) was analyzed with the NeuronJ plug-in from the software NIH ImageJ (Meijering et al., 2004). For statistical comparison, the area under the curve (AUC) of the resulting function was calculated. Quantifications were based on 13–20 cells per animal from 3–6 mice per time-point.

Spine density—To calculate spine density, at least 5 images of randomly selected eGFP-positive dendritic segments of mice from all three genotypes were acquired at 28 and 42 dpi ($n = 3\text{--}4$ mice per time-point and genotype), using a 40x oil immersion objective with a 2.0 digital zoom factor (pixel size, 60 nm); z-stacks were acquired with an interval of 0.7 μm across the entire thickness of the selected dendrite segment.

Spines on the segment were counted using the ImageJ plugin CellCounter. No distinction was made between spine and filopodia. For each segment, the length was measured and number of spines/ μm was then calculated.

Densitometry analysis—Immunoperoxidase stained sections were examined by bright-field microscopy (Carl Zeiss AG, Jena, Germany) using a 10x (N.A. 0.5) dry objective. Images were acquired with an 8-bit color digital camera controlled by AxioVision 4.8 (Carl Zeiss, AG, Jena, Germany). Densitometry analysis of different GABA_AR α -subunits was performed using the MCID M5 software (Imaging Research Inc., Brock University, St Catharines, ON, Canada). Images were digitized on a light box using a CoolSnap digital camera (Photometrics, Tuscon, AZ, USA) with a Micro-Nikkor 55 mm + 12 mm objective (Nikon Corporation). Staining intensity was measured as calibrated relative optical density in nine regions of interest: CA1, CA3, DG, hilus of the hippocampus, somatosensory cortex (layers II–III, IV, V–VI), striatum, ventrobasal complex of the thalamus (or reticular thalamic nucleus for the $\alpha 3$ subunit). Background was measured in the corpus callosum and subtracted.

RNA isolation and quantitative real time-PCR

Adult mice were anaesthetized with isoflurane, decapitated and the brain taken out rapidly on ice. RNA was extracted using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma- Aldrich). cDNA was prepared using random hexamers and SuperScript® III Reverse Transcriptase (Invitrogen). The following primer sequences were used: *Gabra5* fwd 5'-GAA AGG CTG CGG TTT AAG GG-3'; *Gabra5* rev 5'-TGG TAC TGG TTG AGC CTG GA-3'; *GAPDH* fwd 5'-CAT GGC CTT CCG TGT TCC TA-3'; *GAPDH* rev 5'-CCT GCT TCA CCA CCT TCT TGA-3'. Quantitative PCR was performed on a 7900 HT Fast Real Time PCR system (Applied Biosystems). 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (Solis Biotec) was used with the designed primers to amplify the mRNA. Changes in mRNA levels were calculated using the $\Delta\Delta C_t$ Method (Pfaffl et al., 2004) and were normalized to that of *GAPDH* mRNA.

Statistical analyses

Data are presented as mean $\pm$ SEM. Statistical analyses were made using unpaired t-test, one-way ANOVA or two-way ANOVA, as indicated, followed by post-hoc tests where appropriate (Prism software; GraphPad, version 6). Cumulative probability distributions, used for migration distance, were compared using the Kolmogorov-Smirnov test. Statistical significance was set at $P < 0.05$.

Results

Among the various stages of adult neurogenesis in the DG, we investigated here the effects of full, partial, or cell-specific inactivation of *Gabra5* on the migration of newborn GCs into the GCL, their morphological differentiation, and the formation of dendritic spines. For studying the effects of full or partial gene inactivation, we stereotactically injected a retroviral vector encoding eGFP into the dorsal hippocampus of adult wild type, $\alpha 5$ -KO and $\alpha 5$ -het mice and analyzed them morphologically at 7, 14, 28 and 42 dpi. Separate cohorts of wild type, $\alpha 5$ -het, and $\alpha 5^{fl/+}$ mice were injected with a vector encoding Cdk5-eGFP and analyzed at 28 dpi. Cell-specific deletion was achieved by co-injecting a vector encoding Cre-eGFP and a vector encoding mRFP (to visualize dendrites) in $\alpha 5$ -floxed mice, or by co-injecting a vector encoding Cre-mCherry and a vector encoding eGFP or eGFP-Cdk5. With this strategy, we expected to see in the same animal three populations of labeled newborn GCs, either single- or double-transfected. As Cre recombinase is targeted to the nucleus, eGFP or mRFP labeling in double-transfected cells allowed visualizing cell morphology and quantifying dendritic arborization. Control experiments to test whether Cre-expression *per se* might affect dendritic growth were performed in wildtype mice co-injected with a vector encoding eGFP and a vector encoding Cre-mCherry; the dendritic arborization was compared in single- and double-labeled cells at 28 dpi. For all groups, 3–6 mice/time-point were analyzed.

Adult-born GC migration

Post-mitotic GC precursors migrate from the subgranular zone (SGZ), located between the hilus and the GCL, to their final position in the GCL while undergoing morphological and functional differentiation (Kempermann et al., 2004). To quantify migration distance at the

selected time-points, coronal sections through the dorsal hippocampus were stained against GFP and DAPI, allowing the visualization of newborn GCs and their location in the GCL. Their migration distance away from the SGZ was determined as the shortest (orthogonal) distance between the cell center and the SGZ - GCL boundary. At 14 dpi, the soma of eGFP-positive GCs from wild type and $\alpha 5$ -KO mice was located either in the SGZ or a few μm away in the GCL. Strikingly, in $\alpha 5$ -het mice, about 30% labeled cells had migrated in the opposite direction (towards the hilus), whereas the others remained close to the SGZ (Fig. 1A,B). At 14 and 28 dpi, GCs from wild type and $\alpha 5$ -KO mice had migrated further into the GCL, typically in the inner third, whereas at 42 dpi the majority was seen in the middle third of the GCL (Fig. 1A',C-D). No difference between wild type and $\alpha 5$ -KO mice was observed at any time-point. In contrast, eGFP-positive GCs from $\alpha 5$ -het mice migrated less deeply into the GCL, and those that went towards the hilus did not reverse their course. Pair-wise comparison of genotypes using the Kolmogorov-Smirnov test revealed a significant difference between $\alpha 5$ -het mice and either wild type or $\alpha 5$ -KO mice at each time-point analyzed (Fig. 1B-E).

Dendrite development

To determine whether full or partial ablation of the $\alpha 5$ -GABA_AR impairs dendrite growth and branching, as seen in *Gabra4*-null mice (Duveau et al., 2011), we quantified major morphometric parameters (dendrite arbor complexity, primary segment length, total dendritic length, number of terminal branches) in eGFP-positive newborn GCs. At 7 dpi, all cells from the three genotypes had a distinct apical dendrite extending across the GCL (not shown). At 14 dpi, the spine-less dendritic tree extended into the inner molecular layer (not shown), whereas at 28 dpi, distal branches extended almost up to the outer border of the DG, and spine formation could be observed (Fig. 2A). Finally, at 42 dpi, eGFP-positive GCs exhibited a well-developed, mature-like dendritic tree (Fig. 2A). Quantification of the dendritic arbor complexity by Sholl analysis revealed no significant differences between wild type and $\alpha 5$ -KO mice at any time-point, but a progressive increase in dendritic arborization was observed over time. In contrast, $\alpha 5$ -het mice exhibited a marked reduction of dendritic complexity compared to either control or $\alpha 5$ -KO mice (Fig. 2B-E). At each time-point, quantification of the area-under-the-curve by one-way ANOVA confirmed these observations by revealing a significant effect of genotype: 14 dpi: $F_{2,11} = 13.83$, $P = 0.001$; 28 dpi: $F_{2,9} = 18.14$, $P = 0.0007$; 42 dpi: $F_{2,9} = 16.05$, $P = 0.0011$ (Fig. 2B-E).

In all genotypes, the length of the primary segment (from the soma to the first bifurcation) remained constant at all time-points analyzed (Fig. 2F; two-way ANOVA). In contrast, quantification of total dendritic length revealed a significant effect of time ($F_{3,39} = 385.8$, $P < 0.0001$) and genotype ($F_{2,39} = 63.25$, $P < 0.0001$), as well as a significant interaction ($F_{6,39} = 10.31$, $P < 0.0001$). *Post-hoc* analysis revealed that eGFP-positive GCs from $\alpha 5$ -het mice exhibited a significant reduction in total dendritic length compared to wild type control or $\alpha 5$ -KO at 28 ($P < 0.001$) and 42 dpi ($P < 0.001$); however, no differences could be detected between wild type and $\alpha 5$ -KO mice (Fig. 2G). The significant time x genotype interaction likely indicates that dendrite growth in GCs from $\alpha 5$ -het mice is retarded compared to control and $\alpha 5$ -KO mice. Additionally, quantification of number of terminal branches revealed a significant effect of time ($F_{3,41} = 36.44$; $P < 0.0001$) and genotype ($F_{2,41} = 12.77$;

$P < 0.0001$), but no interaction, indicating a similar time-dependent evolution of branching. However, *post-hoc* analysis indicated that, whereas newborn neurons from wild type and $\alpha 5$ -KO mice did not differ from each other, newborn GCs from $\alpha 5$ -het mice had a reduced number of terminal branches at 28 dpi ($P < 0.001$) (Fig. 2H).

The results so far unexpectedly show that constitutive *Gabra5* inactivation caused no effect on migration and dendritic development in adult-born GCs, whereas a partial deletion had strong and enduring effects on these processes that are essential for their proper functional integration.

Next, we compared these results with the effects of single-cell *Gabra5* deletion on dendritic complexity of mRFP-positive (wild type) and double-transfected GCs (Fig. 3A–B). Starting with migration (Fig. 1F–H), we observed a deficit for mutant cells, co-expressing Cre-recombinase and mRFP compared to wild type cells, which was significant at each of the three time-points analyzed, pointing to a cell-autonomous effect.

Quantification of dendritic maturation by Sholl analysis (Fig. 3C–E) revealed no difference at 14 dpi between Cre-positive GCs (with deletion of *Gabra5*) and Cre-negative GCs, confirming our results above (Fig. 2C) that $\alpha 5$ -GABA_ARs are not essential for initial dendritic growth. However, at 28 dpi, the dendritic arborization of mutant cells in the molecular layer was less complex compared to Cre-negative cells, as seen by comparing the area-under-the-curves (unpaired t-test, $t_{42} = 2.14$; $P = 0.038$) (Fig. 3D). The difference remained evident at 42 dpi, in particular in the inner two-thirds of distal branches (on average, between 40 and 200 μm away from the soma) in mutant cells compared to Cre-negative cells (unpaired t-test, $t_{32} = 2.22$; $P = 0.034$; Fig. 3E).

The remaining dendritic parameters measured (length of the primary segment, total dendritic length, number of branches) exhibited similar differences as those seen between wild type and $\alpha 5$ -het mice. Thus, the length of the primary segment was not affected by the mutation (Fig. 3F), whereas statistical analysis of total dendritic length showed a significant effect of time ($F_{2,12} = 8.92$; $P = 0.0042$) and genotype ($F_{1,12} = 8.58$; $P = 0.0126$), but without an interaction. *Post-hoc* analysis revealed that mutant cells had a reduced total dendritic length at 28 dpi (Fig. 3G). Further, analysis of the number of terminal branches showed a significant effect of time ($F_{2,12} = 5.78$; $P = 0.0175$) and genotype ($F_{1,12} = 11.79$; $P = 0.005$), but no interaction. Mutant cells had a reduced number of terminal branches at 28 dpi (Fig. 3H). The shorter total dendritic length of mRFP-positive cells in wild type (Fig. 3G) mice compared to the eGFP-positive cells in wild type and $\alpha 5$ -mutant mice (see Fig. 2G) likely reflects the lower signal-to-noise ratio obtained with mRFP labeling; however, these should not affect the conclusion, since we compared mutant (Cre-positive) and Cre-negative cells in the same tissue sections.

To verify that Cre expression *per se* does not affect dendrite development due to possible toxicity, control experiments were performed in which wild type mice were injected with a mixture of retroviruses encoding eGFP and mCherry-Cre. Sholl analysis of dendrites from granule cells single-labeled with GFP and double-labeled with GFP and mCherry revealed no significant difference for all parameters analyzed (number of intersections, number of

terminal branches, total dendritic length), indicating that Cre expression in wild-mice does not affect dendritic development of adult-born granule cells (data not shown).

Taken together, these data suggest that the effects seen in adult-born GCs of $\alpha 5$ -het mice are due to cell autonomous action of $\alpha 5$ -GABA_ARs controlling their migration and dendrite development. Intriguingly, the deficits were not more severe upon Cre-mediated inactivation of the *Gabra5* gene, suggesting that a partial deficit in $\alpha 5$ -GABA_ARs can cause a functional imbalance that is not worsened by the complete elimination of the receptor.

Spine density

Extrasynaptic $\alpha 5$ -GABA_ARs have been postulated to be well positioned for modulating the spread of excitation from glutamatergic synapses formed on spines. It was, therefore, of major interest to determine whether ablation of $\alpha 5$ -GABA_ARs would affect spine formation. Quantification of spine density (number of spines per μm dendrite) at 28 dpi in intermediate and distal dendritic segments in the ML of eGFP-positive neurons in wild type (1.37 ± 0.07 spines/ μm), $\alpha 5$ -KO (1.39 ± 0.03 spines/ μm) and $\alpha 5$ -het (1.41 ± 0.05 spines/ μm) mice revealed no difference between genotypes. Likewise, at 42 dpi spine density was uniform across genotypes (wild type: 1.43 ± 0.01 spines/ μm ; $\alpha 5$ -KO: 1.48 ± 0.1 spines/ μm ; $\alpha 5$ -het: 1.57 ± 0.08 spines/ μm) (Fig. 4A–B). Further, two-way ANOVA analysis revealed no significant effect of time ($F_{1,11} = 4.79$; $P = 0.051$), indicating that maximal spine density is achieved already at 28 dpi. We did not attempt to distinguish among spine types based on their morphology, in particular because we know from previous work (Deprez et al., 2015) that spine filopodia might not be detectable using GFP-immunofluorescence in immature cells. However, these data are in line with our previous results in *Gabra2*- and *Gabra4*-null mice (Duveau et al., 2011) and confirm that spine formation in GCs might be governed by extrinsic influences from the perforant path (Frotscher et al., 2000). Further, similarly to CA1 pyramidal cells (Bonin et al., 2007), they imply that adult-born GCs from $\alpha 5$ -KO and $\alpha 5$ -het mice might be hyper-excitable.

Compensatory changes in GABA_AR α subunit expression in the hippocampal formation

Considering the lack of phenotype seen so far in adult-born GCs from $\alpha 5$ -KO mice, we wondered whether the absence of $\alpha 5$ -GABA_ARs might be compensated for by overexpression of another α subunit variant. Although immunohistochemistry is not a quantitative method, it allows differentiating the relative abundance of a given protein on a regional or even cellular basis. Therefore, expecting that a compensatory increase might be most prominent in regions enriched in $\alpha 5$ -GABA_ARs, we compared the regional distribution of the $\alpha 1$ – $\alpha 4$ subunits in brain sections from naïve wild type, $\alpha 5$ -KO, and $\alpha 5$ -het littermates, as detected by immunoperoxidase staining. For each subunit, we measured by densitometry analysis the relative staining intensity in the dorsal hippocampus (CA1, CA3, DG, hilus), somatosensory cortex (layers II–III, IV, V–VI), striatum, and thalamus (ventrobasal complex or reticular nucleus) (Fig. 5; Table 2). First, this experiment allowed validating the specificity of the antibody against the $\alpha 5$ subunit; second, it demonstrates that the protein is not detectable in brain tissue from $\alpha 5$ -KO mice (Fig. 5B).

For each subunit, we observed distinct, genotype- and region-specific changes, illustrated for the $\alpha 1$ and $\alpha 5$ subunit (Fig. 5A–B), as determined by two-way ANOVA analysis ($\alpha 1$ subunit: genotype, $F_{2,8} = 49.37$, $P < 0.0001$; region, $F_{2,8} = 20.53$, $P < 0.0001$; $\alpha 2$ subunit: genotype, $F_{2,8} = 5.82$, $P = 0.004$, region, $P < 0.0001$; $\alpha 3$ subunit: genotype, $F_{2,8} = 26.77$, $P < 0.0001$; region, $F_{2,8} = 81.02$, $P < 0.0001$; $\alpha 4$ subunit: genotype, $F_{2,8} = 26.39$, $P < 0.0001$; region, $F_{2,8} = 91.36$, $P < 0.0001$; $\alpha 5$ subunit: genotype, $F_{2,8} = 9.27$, $P < 0.0034$; region, $F_{2,8} = 81.22$, $P < 0.0001$). However, post-hoc tests only confirmed the increased staining intensity of the $\alpha 1$ subunit in hippocampus and cerebral cortex of $\alpha 5$ -KO mice, along with a reduction in $\alpha 3$ subunit staining in the thalamic reticular nucleus (Fig. 5A, C), reflecting the inter-individual variability in staining intensity. Strikingly, the $\alpha 5$ subunit-immunoreactivity was not decreased in $\alpha 5$ -het mice, even in the CA1 and CA3 areas, where it is most strongly expressed (Fig. 5B, C). To understand the basis of this unexpected observation, we determined the expression levels of $\alpha 5$ subunit mRNA by qPCR analysis in the brain of wild type, $\alpha 5$ -het and $\alpha 5$ -KO mice. In the latter, the $\alpha 5$ subunit mRNA was below detection level; in $\alpha 5$ -het mice, the levels were $77.9 \pm 6\%$ of wild type ($N=3$ mice/genotype). Therefore, the inactivation of one allele is partially compensated for on the transcriptional, and likely translational level.

Taken together, these observations suggest that constitutive *Gabra5* deletion induces compensatory increases in the expression profile of other GABA_AR subunits; whereas in $\alpha 5$ -het mice, a single allele appears sufficient to maintain $\alpha 5$ -subunit expression throughout the brain. The resolution of this approach was insufficient to determine whether these changes also occur in neuronal progenitor cells of the SGZ.

Overexpression of Cdk5 induces partial phenotypic rescue in $\alpha 5$ -het mice

Altered migration of adult-born GCs towards the hilus and impaired growth of dendrites, as seen in $\alpha 5$ -het mice, was reminiscent of the phenotype reported upon silencing of Cdk5 expression (Jessberger et al., 2008). Therefore, we wondered whether Cdk5 signaling might be affected in GCs of $\alpha 5$ -het mice and tested whether infecting neural progenitor cells with a retrovirus encoding eGFP-tagged Cdk5 might restore their phenotype. The effects were assessed in wild type and $\alpha 5$ -het mice at 28 dpi and the results included in Figure 1D (migration) and Figure 2D, F–H (dendrite development). Strikingly, the migration deficit selectively observed in $\alpha 5$ -het mice was completely rescued upon overexpression of Cdk5, without any effects in wild type (Fig. 1D). Dendrite growth was differentially affected in $\alpha 5$ -het and wild type mice by Cdk5. In the former, Sholl analysis revealed a trend towards a more complex tree, while in the latter they were significantly reduced (28 dpi; one-way ANOVA, $F_4 = 7.69$, $P < 0.0001$; Fig. 2D). These differential alterations were also evident when quantifying total dendritic length (28 dpi; one-way ANOVA, $F_4 = 20.84$, $P < 0.001$); it was reduced in both genotypes upon Cdk5 expression compared to wild type levels (Fig. 2G). Nevertheless, the number of terminal branches recovered to normal levels upon overexpression of Cdk5 in $\alpha 5$ -het mice ($F_4 = 13.25$, $P < 0.001$) (Fig. 2H), suggesting that Cdk5 exerts multiple effects on dendritic growth.

These findings raised the issue whether a single-cell inactivation of *Gabra5* in a heterozygous background (i.e., in $\alpha 5^{fl/+}$ mice) would reproduce the effects seen in either $\alpha 5$ -

het or in $\alpha 5^{fl/fl}$ mice. To this end, we analyzed two groups of $\alpha 5^{fl/+}$ mice (N=6), injected with retroviruses expressing mCherry-Cre and either eGFP or eGFP-Cdk5; and investigated after 28 DIV the dendrite morphology of single-labeled cells (expressing eGFP or eGFP-Cdk5) or double-labeled cells (expressing mCherry-Cre in the nucleus along with eGFP or eGFP-Cdk5). Sholl analysis did not reveal a significant difference in dendrite complexity between the four groups (28 dpi; one-way ANOVA, $F_3 = 0.4663$, $P = 0.7068$), a result confirmed by quantification of total dendritic length ($F_3 = 1.302$, $P = 0.284$) and number of terminal branches ($F_3 = 1.978$, $P = 0.1292$; Fig. 3I–K). Expression of mCherry-Cre was very distinct and the same construct (and virus) have been used in experiments yielding positive effects, making it unlikely that Cre-recombinase was inactive in this assay. Therefore, we concluded that targeted deletion of one *Gabra5* allele in adult-born neurons of $\alpha 5^{fl/+}$ mice is not sufficient to impair dendritic growth, and that overexpression of Cdk5 in this assay did not cause detectable effects.

Discussion

To our knowledge, despite the intense scrutiny given to $\alpha 5$ -GABA_ARs for their role in regulating neuronal excitability and cognitive performance (see Introduction), their contribution to adult neurogenesis has not yet been investigated. The surprising lack of phenotype in global $\alpha 5$ -KO mice might be tentatively explained by the activation of compensatory mechanisms. Although causality was not demonstrated, the up-regulation of $\alpha 1$ -GABA_ARs, and to lesser extent $\alpha 4$ -GABA_ARs – which both have also been observed by (Engin et al., 2015) upon targeted *Gabra5* deletion in the hippocampal formation – suggested global changes in GABA_AR expression selectively in $\alpha 5$ -KO mice. In contrast to these mice, the results show that a partial inactivation of *Gabra5* causes severe and enduring alterations of the migration and dendrite development of adult-born GCs, although the levels of $\alpha 5$ subunit immunoreactivity were not decreased in the hippocampal formation of $\alpha 5$ -het mice. These alterations are, for a large part, cell-autonomous, as shown by conditional deletion of *Gabra5* selectively in newborn GCs. Remarkably, despite the evidence that silencing $\alpha 5$ -GABA_ARs leads to hyperexcitability (of mature neurons) due to reduced tonic inhibition (Glykys and Mody, 2006; Bonin et al., 2007), we observed a deficit, rather than an excess, of migration and dendrite growth/branching, reminiscent of that reported upon silencing of Cdk5 (Jessberger et al., 2008). Indeed, overexpression of Cdk5 could prevent some of these deficits, suggesting that Cdk5 signaling might cooperate with $\alpha 5$ -GABA_ARs to regulate dendritic growth.

Effects of *Gabra5* gene deletion on GABA_AR subunit expression

In analogy with previous studies of targeted *Gabra1-Gabra4* gene deletions (see (Fritschy and Panzanelli, 2014) for review), and with previous reports on mice carrying a chromosomal deletion encompassing *Gabra5* (Fritschy et al., 1997; Fritschy et al., 1998), a key premise of the present work is that *Gabra5* targeting results in the complete loss of $\alpha 5$ -GABA_ARs. We show that the $\alpha 5$ subunit mRNA and protein is undetectable in $\alpha 5$ -KO mice, as expected and previously shown by (Collinson et al., 2002) in their $\alpha 5$ -KO mice. Furthermore, electrophysiological studies of $\alpha 5$ -KO mice provided ample evidence for the

absence of functional deficits caused by the absence of the corresponding GABA_AR (Glykys and Mody, 2006; Bonin et al., 2007).

However, as the expression pattern of GABA_AR subunits by neural precursor cells is only poorly established, a major issue to be resolved is whether inactivation of $\alpha 5$ -GABA_ARs affects newborn cells directly or indirectly by changing the properties of neuronal networks in the DG (and its afferent/efferent connections). Finally, it is not known whether $\alpha 5$ -GABA_ARs expressed in developing GCs are post- or extrasynaptic. In view of the convergent, but not identical, phenotype seen in $\alpha 5$ -het mice and conditional knockout $\alpha 5^{\text{fl/fl}}$ GCs, we conclude that both direct and indirect effects contribute to the altered migration behavior and dendrite formation. Accordingly, the lack of phenotype observed in $\alpha 5$ -KO mice can be viewed as evidence for the existence of compensatory changes affecting the intrinsic properties of developing GCs and/or their environment.

An important factor for the interpretation of our results is whether immunohistochemical staining followed by densitometry analysis is a suitable method to assess regional (or even cellular) changes in the abundance of a given α subunit variant (and, by inference, of the corresponding GABA_AR subtype(s) in the brain of $\alpha 5$ -mutant mice. Immunohistochemistry is semi-quantitative, at best, because multiple factors contribute to signal intensity (antibody affinity, tissue penetration, strength of enzymatic reaction, to name just a few) and because tissue fixation strongly affects the apparent distribution of epitopes. Nevertheless, in previous studies, we have been able to detect regional alterations in immunohistochemical signals that corresponded to major functional and/or behavioral alterations. Most relevant for our conclusions is the lack of decreased $\alpha 5$ subunit-immunoreactivity in $\alpha 5$ -het mice, which strongly contrasts with the ~30% reduction observed regionally in brain tissue expressing $\alpha 5$ (H105R) point-mutated subunit, confirmed by autoradiography (Crestani et al., 2002; Prut et al., 2010). Therefore, we conclude that the $\alpha 5$ subunit protein is not globally reduced in the hippocampal formation of $\alpha 5$ -het mice, and that a possible compensation occurs selectively in $\alpha 5$ -KO mice by overexpression of the $\alpha 1$, and to a lesser extent, $\alpha 4$ subunits.

Previous studies had already shown in the hippocampus that up-regulation of the δ subunit partly compensates for the loss of tonic inhibition mediated by $\alpha 5$ -GABA_AR (Glykys and Mody, 2006; Glykys et al., 2008). However, this change was not sufficient to prevent network hyperexcitability in CA1. Here, we observed a marked increase in $\alpha 1$ subunit and a moderate increase in $\alpha 4$ subunit immunoreactivity, with a significant effect of genotype, but no pairwise difference in post-hoc tests among the various subregions analyzed. As the $\alpha 1$ subunit is present in both, extra- and postsynaptic receptors, its upregulation in $\alpha 5$ -KO mice might reflect an increase in tonic as well as phasic inhibition. In this study, it was not possible to determine whether the upregulation of $\alpha 1$ subunit immunoreactivity detected in the DG also occurs in immature GCs. However, since both $\alpha 1$ and $\alpha 4$ subunits contribute to extrasynaptic GABA_ARs in the DG (Glykys et al., 2008; Herd et al., 2008), their global increase in the DG likely contributes to maintain tonic inhibition in $\alpha 5$ -KO mice. In contrast, the $\alpha 2$ subunit immunoreactivity remains largely unchanged in $\alpha 5$ -KO mice. As $\alpha 2$ -GABA_ARs are the most abundant subtype in the hippocampal formation, these observations might explain the increased excitability of CA1 pyramidal cells and networks (Bonin et al., 2007).

Besides the alterations in GABA_AR subunit-IR analyzed here, compensatory changes in neuronal circuits of $\alpha 5$ -KO mice could affect many other molecules, notably voltage-gated ion channels (Brickley et al., 2001). Therefore, one might conclude from the present results that adult neurogenesis is protected from major disturbances in $\alpha 5$ -KO mice by activation of selective compensatory mechanisms. Given that the $\alpha 5$ subunit is strongly expressed during fetal development, the purpose of these compensatory mechanisms is likely to preserve proper CNS development.

Thus, the strong phenotype observed in $\alpha 5$ -het mice, especially when compared to the full knockout, suggests that partial preservation of $\alpha 5$ -GABA_ARs is sufficient to prevent the activation of compensatory mechanisms. This conclusion most likely also holds upon conditional $\alpha 5$ subunit inactivation in GCs from $\alpha 5^{fl/fl}$ mice, implying that loss-of-function of $\alpha 5$ -GABA_ARs caused the deficit in migration and dendritic growth. Therefore, it was unexpected that the $\alpha 5$ -subunit immunoreactivity would not be partially decreased in $\alpha 5$ -het mice. Particularly, in view of the fact that $\alpha 5$ (H105R) knock-in mice, which lack about 30% $\alpha 5$ -GABA_ARs (Balic et al., 2009), exhibit decreased immunohistochemical staining for the $\alpha 5$ subunit in the hippocampal formation (Crestani et al., 2002; Prut et al., 2010). The most straightforward explanation for this discrepancy is that adult-born, but not mature, GCs in $\alpha 5$ -het mice have a reduced expression of the $\alpha 5$ subunit. As a corollary, one might speculate that $\alpha 5$ (H105R) knock-in mice, which exhibit multiple signs of hippocampus (and amygdala) dysfunction (see Introduction), also have a deficit in adult neurogenesis.

Effect of partial and single-cell deletion of $\alpha 5$ -GABA_ARs

Disruption of tonic inhibition by the deletion of the $\alpha 4$ -GABA_ARs strongly affects neural precursor cell proliferation, as well as migration and dendritic development of newborn neurons (Duveau et al., 2011). In these cells, patch-clamp recordings performed at 14–21 dpi revealed a complete loss of tonic inhibition, indicating the prime role of $\alpha 4$ -GABA_ARs in developing GCs for mediating extrasynaptic effects of GABA. Consequently, $\alpha 5$ -GABA_ARs might either be expressed later (which is unlikely), or contribute to slow or fast phasic inhibition in developing GCs. Ivy/neurogliaform cells are the main interneuron subtype that forms initial connections onto newborn GCs, and exert a form of slow transmission modulated by GABA transporters (Markwardt et al., 2011). While the GABA_AR subtype mediating these effects has not been identified, $\alpha 5$ -GABA_ARs are a likely candidate, based on the kinetics of the responses evoked upon stimulation of Ivy cells. The presence of $\alpha 5$ -GABA_AR in neural stem cells of the SGZ has been suggested in a study of conditional *Gabrg2* gene deletion in mice, showing that a zolpidem-insensitive GABA_AR subtype controls proliferation of quiescent radial glial-like NSCs and regulates differentiation of astrocytes (Song et al., 2012). Therefore, to fully understand the role of $\alpha 5$ -GABA_ARs in the SGZ, it will be essential to determine their specific function and subcellular distribution.

The phenotype observed upon conditional *Gabra5* deletion, either in $\alpha 5$ -het mice or in mutant conditional KO GCs from $\alpha 5^{fl/fl}$ mice, points to a cell-autonomous function of $\alpha 5$ -GABA_ARs, promoting neuronal development of newborn neurons, and, therefore, confirming their early expression. Failure to initiate proper migration, notably in the correct direction, underscores the importance of GABAergic transmission for positioning of adult-

born neurons in the GCL. Previous evidence showed that this mechanism requires activation of Cdk5 (Jessberger et al., 2008), making it plausible that signaling through $\alpha 5$ -GABA_ARs acts upstream of Cdk5, or, at the least, that the two signaling pathways can act on the same effector. This possibility was further supported from evidence that Cdk5 activation is required for proper dendritic growth, via activation of focal adhesion kinase and phosphorylation of the semaphorin receptors neuropilin 1 and 2 (Ng et al., 2013). There is only limited experimental evidence about the mechanisms by which Cdk5 modulate neuronal migration (reviewed in (Jessberger et al., 2009)), although phosphorylation of cytoskeletal proteins involved in nucleokinesis appears plausible. A major issue to be resolved, however, is to determine whether the levels of active (i.e. phosphorylated) Cdk5 are changed in newborn cells of $\alpha 5$ -het and wild type mice.

The less severe phenotype observed in mutant conditional KO GCs from $\alpha 5^{fl/fl}$ mice compared to $\alpha 5$ -het mice points to cell non-autonomous mechanisms regulating the maturation of adult-born neurons under the control of $\alpha 5$ -GABA_ARs. It is conceivable, of course, that enhanced network hyperexcitability in $\alpha 5$ -het mice, might impair GABAergic control of neuronal maturation or disturb the balance between excitation and inhibition that is crucial for proper development and functional maturation of adult-born neurons (Dieni et al., 2013).

In conclusion, the present study demonstrates that a partial, but not a full, inactivation of $\alpha 5$ -GABA_ARs in mice causes enduring deficits in the maturation of adult-born GCs. When combined with the wealth of evidence that weakening $\alpha 5$ -GABA_AR-mediated transmission improves cognitive performance, in particular in conditions of impaired excitatory/inhibitory balance, such as Down syndrome (reviewed in (Rudolph and Möhler, 2014)), these data shed a note of caution about possible negative consequences arising from chronic pharmacological treatment with negative allosteric modulator acting selectively on $\alpha 5$ -GABA_ARs.

Acknowledgments

JMF was supported by the Swiss National Science Foundation (Grant 310030_146120). UR was supported by award number R01GM086448 from the National Institute of General Medical Sciences of the National Institute of Health and award numbers R01MH080006 and R01MH095905 of the National Institute of Mental Health. We are grateful to Dr. Sebastian Jessberger (University of Zurich) for generously providing the Cdk5 construct and the Cre-mCherry retroviral vector, Claire de Groot and Yuen-Chen Tsai for help with virus production and qPCR analysis, and to Dr. Elif Engin for critical comments on the manuscript.

References

- Balic E, Rudolph U, Fritschy JM, Mohler H, Benke D. The $\alpha 5$ (H105R) mutation impairs $\alpha 5$ selective binding properties by altered positioning of the $\alpha 5$ subunit in GABA_A receptors containing two distinct types of α subunits. *J Neurochem.* 2009; 110:244–254. [PubMed: 19457072]
- Belelli D, Harrison N, Maguire J, Macdonald R, Walker M, Cope D. Extrasynaptic GABA_A receptors: form, pharmacology, and function. *J Neurosci.* 2009; 29:12757–12763. [PubMed: 19828786]
- Bonin RP, Martin LJ, Macdonald JF, Orser BA. $\alpha 5$ -GABA_A receptors regulate the intrinsic excitability of mouse hippocampal pyramidal neurons. *J Neurophysiol.* 2007; 98:2244–2254. [PubMed: 17715197]

- Brickley SG, Revilla V, Cull-Candy SG, Wisden W, Farrant M. Adaptive regulation of neuronal excitability by a voltage-independent potassium conductance. *Nature*. 2001; 409:88–92. [PubMed: 11343119]
- Caraiscos VB, Elliott EM, You-Ten KE, Cheng VY, Belelli D, Newell JG, Jackson MF, Lambert JJ, Rosahl TW, Wafford KA, MacDonald JF, Orser BA. Tonic inhibition in mouse hippocampal CA1 pyramidal neurons is mediated by $\alpha 5$ subunit-containing γ -aminobutyric acid type A receptors. *Proc Natl Acad Sci USA*. 2004; 101:3662–3667. [PubMed: 14993607]
- Collinson N, Kuenzi FM, Jarolimek W, Maubach KA, Cothliff R, Sur C, Smith AD, Otu FM, Howell O, Attack JR, McKernan RM, Seabrook GR, Dawson GR, Whiting PJ, Rosahl TW. Enhanced learning and memory and altered GABAergic synaptic transmission in mice lacking the $\alpha 5$ subunit of the GABA_A receptor. *J Neurosci*. 2002; 22:5572–5580. [PubMed: 12097508]
- Crestani F, Keist R, Fritschy JM, Benke D, Vogt K, Prut L, Bluethmann H, Mohler H, Rudolph U. Trace fear conditioning involves hippocampal $\alpha 5$ GABA_A receptors. *Proc Natl Acad Sci USA*. 2002; 99:8980–8985. [PubMed: 12084936]
- Dawson G, Maubach K, Collinson N, Cobain M, Everitt B, MacLeod A, Choudhury H, McDonald L, Pillai G, Rycroft W, Smith A, Sternfeld F, Tattersall F, Wafford K, Reynolds D, Seabrook G, Attack J. An inverse agonist selective for $\alpha 5$ subunit-containing GABA_A receptors enhances cognition. *J Pharmacol Exp Ther*. 2006; 316:1335–1345. [PubMed: 16326923]
- Deprez F, Pallotto M, Vogt F, Grabiec M, Virtanen M, Tyagarajan S, Panzanelli P, Fritschy J. Postsynaptic gephyrin clustering controls the development of adult-born granule cells in the olfactory bulb. *J Comp Neurol*. 2015; 523:1998–2016. [PubMed: 25772192]
- Dieni C, Chancey J, Overstreet-Wadiche L. Dynamic functions of GABA signaling during granule cell maturation. *Front Neural Circuits*. 2013; 6:113. [PubMed: 23316139]
- Duveau V, Laustela S, Barth L, Gianolini F, Vogt KE, Keist R, Chandra D, Homanics GE, Rudolph U, Fritschy JM. Spatiotemporal specificity of GABA_A receptor-mediated regulation of adult hippocampal neurogenesis. *Eur J Neurosci*. 2011; 34:362–373. [PubMed: 21722213]
- Engin E, Zarnowska E, Benke D, Tsvetkov E, Sigal M, Keist R, Bolshakov V, Pearce R, Rudolph U. Tonic inhibitory control of dentate gyrus granule cells by $\alpha 5$ -containing GABA_A receptors reduces memory interference. *J Neurosci*. 2015; 35:13698–13712. [PubMed: 26446222]
- Fritschy J, Panzanelli P. GABAA receptors and plasticity of inhibitory neurotransmission in the central nervous system. *Eur J Neurosci*. 2014; 39:1845–1865. [PubMed: 24628861]
- Fritschy JM, Mohler H. GABA_A-receptor heterogeneity in the adult rat brain: differential regional and cellular distribution of seven major subunits. *J Comp Neurol*. 1995; 359:154–194. [PubMed: 8557845]
- Fritschy JM, Johnson DK, Mohler H, Rudolph U. Independent assembly and subcellular targeting of GABA_A receptor subtypes demonstrated in mouse hippocampal and olfactory neurons in vivo. *Neurosci Lett*. 1998; 249:99–102. [PubMed: 9682826]
- Fritschy JM, Benke D, Johnson DK, Mohler H, Rudolph U. GABA_A-receptor α -subunit is an essential prerequisite for receptor formation in vivo. *Neuroscience*. 1997; 81:1043–1053. [PubMed: 9330366]
- Frotscher M, Drakew A, Heimrich B. Role of afferent innervation and neuronal activity in dendritic development and spine maturation of fascia dentata granule cells. *Cereb Cortex*. 2000; 10:946–951. [PubMed: 11007545]
- Gill K, Grace A. The role of $\alpha 5$ GABA_A receptor agonists in the treatment of cognitive deficits in schizophrenia. *Curr Pharm Des*. 2014; 20:5069–5076. [PubMed: 24345268]
- Glykys J, Mody I. Hippocampal network hyperactivity after selective reduction of tonic inhibition in GABA_A receptor $\alpha 5$ subunit-deficient mice. *J Neurophysiol*. 2006; 95:2796–2807. [PubMed: 16452257]
- Glykys J, Mann EO, Mody I. Which GABA_A receptor subunits are necessary for tonic inhibition in the hippocampus? *J Neurosci*. 2008; 28:1421–1426. [PubMed: 18256262]
- Hauser J, Rudolph U, Keist R, Möhler H, Feldon J, Yee B. Hippocampal $\alpha 5$ subunit-containing GABA_A receptors modulate the expression of prepulse inhibition. *Mol Psychiatry*. 2005; 10:201–207. [PubMed: 15263904]

- Herd MB, Haythornthwaite AR, Rosahl TW, Wafford KA, Homanics GE, Lambert JJ, Belelli D. The expression of GABA_A β subunit isoforms in synaptic and extrasynaptic receptor populations of mouse dentate gyrus granule cells. *J Physiol (Lond)*. 2008; 586:989–1004. [PubMed: 18079158]
- Jessberger S, Gage F, Eisch A, Lagace D. Making a neuron: Cdk5 in embryonic and adult neurogenesis. *Trends Neurosci*. 2009; 32:575–582. [PubMed: 19782409]
- Jessberger S, Aigner S, Clemenson G, Toni N, Lie D, Karalay O, Overall R, Kempermann G, Gage F. Cdk5 regulates accurate maturation of newborn granule cells in the adult hippocampus. *PLoS Biol*. 2008; 6:e272. [PubMed: 18998770]
- Kempermann G, Jessberger S, Steiner B, Kronenberg G. Milestones of neuronal development in the adult hippocampus. *Trends Neurosci*. 2004; 27:447–452. [PubMed: 15271491]
- Liu X, Wang Q, Haydar TF, Bordey A. Nonsynaptic GABA signaling in postnatal subventricular zone controls proliferation on GFAP-expressing progenitors. *Nature Neurosci*. 2005; 8:1179–1187. [PubMed: 16116450]
- Markwardt SJ, Dieni CV, Wadiche JI, Overstreet-Wadiche L. Ivy/neurogliaform interneurons coordinate activity in the neurogenic niche. *Nat Neurosci*. 2011; 14:1407–1409. [PubMed: 21983681]
- Martin B, Corbin J, Huntsman M. Deficient tonic GABAergic conductance and synaptic balance in the fragile X syndrome amygdala. *J Neurophysiol*. 2014; 112:890–902. [PubMed: 24848467]
- Martínez-Cué C, Martínez P, Rueda N, Vidal R, García S, Vidal V, Corrales A, Montero J, Pazos Á, Flórez J, Gasser R, Thomas A, Honer M, Knoflach F, Trejo J, Wettstein J, Hernández M. Reducing GABA_A $\alpha 5$ receptor-mediated inhibition rescues functional and neuromorphological deficits in a mouse model of Down syndrome. *J Neurosci*. 2013; 33:3953–3966. [PubMed: 23447605]
- Meijering E, Jacob M, Sarria JCF, Steiner P, Hirling H, Unser M. Design and validation of a tool for neurite tracing and analysis in fluorescence microscopy images. *Cytometry Part A*. 2004; 58:167–176.
- Ng T, Ryu J, Sohn J, Tan T, Song H, Ming G, Goh E. Class 3 semaphorin mediates dendrite growth in adult newborn neurons through Cdk5/FAK pathway. *PLoS ONE*. 2013; 8:e65572. [PubMed: 23762397]
- Notter T, Panzanelli P, Pfister S, Mircsof D, Fritschy J. A protocol for concurrent high-quality immunohistochemical and biochemical analyses in adult mouse central nervous system. *Eur J Neurosci*. 2014; 39:165–175. [PubMed: 24325300]
- Pfaffl M, Tichopad A, Prgomet C, Neuvians T. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pairwise correlations. *Biotechnol Lett*. 2004; 26:509–515. [PubMed: 15127793]
- Prenosil GA, Schneider Gasser EM, Rudolph U, Keist R, Fritschy JM, Vogt KE. Specific subtypes of GABA_A receptors mediate phasic and tonic forms of inhibition in hippocampal pyramidal neurons. *J Neurophysiol*. 2006; 96:846–857. [PubMed: 16835366]
- Prut L, Prenosil GA, Willadt S, Vogt K, Fritschy JM, Crestani F. A reduction in hippocampal GABA_A receptor $\alpha 5$ subunits disrupts the memory for location of objects in mice. *Genes Brain Behav*. 2010; 9:478–488. [PubMed: 20180861]
- Rudolph U, Möhler H. GABA_A receptor subtypes: Therapeutic potential in Down syndrome, affective disorders, schizophrenia, and autism. *Annu Rev Pharmacol Toxicol*. 2014; 54:483–507. [PubMed: 24160694]
- Serwanski DR, Miralles CP, Christie SB, Mehta AK, Li X, De Blas AL. Synaptic and nonsynaptic localization of GABA_A receptors containing the $\alpha 5$ subunit in the rat brain. *J Comp Neurol*. 2006; 499:458–470. [PubMed: 16998906]
- Song J, Zhong C, Bonaguidi MA, Sun GJ, Hsu D, Gu Y, Meletis K, Huang ZJ, Ge S, Enikolopov G, Deisseroth K, Luscher B, Christian KM, Ming GL, Song H. Neuronal circuitry mechanism regulating adult quiescent neural stem-cell fate decision. *Nature*. 2012; 489:150–154. [PubMed: 22842902]
- Sur C, Quirk K, Dewar D, Atack J, McKernan R. Rat and human hippocampal $\alpha 5$ subunit-containing γ -aminobutyric acid_A receptors have $\alpha 5 \beta 3 \gamma 2$ pharmacological characteristics. *Mol Pharmacol*. 1998; 54:928–933. [PubMed: 9804628]

- Vargas-Caballero M, Martin LJ, Salter MW, Orser BA, Paulsen O. $\alpha 5$ Subunit-containing GABA_A receptors mediate a slowly decaying inhibitory synaptic current in CA1 pyramidal neurons following Schaffer collateral activation. *Neuropharmacol.* 2010; 58:668–675.
- Wang D, Zurek A, Lecker I, Yu J, Abramian A, Avramescu S, Davies P, Moss S, Lu W, Orser B. Memory deficits induced by inflammation are regulated by $\alpha 5$ -subunit-containing GABA_A receptors. *Cell Rep.* 2012; 2:488–496. [PubMed: 22999935]
- Yee B, Hauser J, Dolgov V, Keist R, Möhler H, Rudolph U, Feldon J. GABA_A receptors containing the $\alpha 5$ subunit mediate the trace effect in aversive and appetitive conditioning and extinction of conditioned fear. *Eur J Neurosci.* 2004; 20:1928–1936. [PubMed: 15380015]
- Zarnowska ED, Keist R, Rudolph U, Pearce RA. GABA_A receptor $\alpha 5$ subunits contribute to GABA_{A,slow} synaptic inhibition in mouse hippocampus. *J Neurophysiol.* 2009; 101:1179–1191. [PubMed: 19073796]

Figure 1.

Migration distance of newborn GCs in wild type, $\alpha 5$ -KO and $\alpha 5$ -het mice. **A, A'**. Representative images illustrating the localization of eGFP-positive soma of newborn GCs at 7 dpi and 42 dpi in the GCL (blue) of wild type and mutant mice. **B–E**. Cumulative distribution analysis of the migration distance in the GCL of eGFP-positive GCs at 7 dpi (B), 14 dpi (C), 28 dpi (D) and 42 dpi (E). No significant difference was observed between wild type and $\alpha 5$ -KO newborn GCs, whereas newborn GCs of $\alpha 5$ -het mice migrate significantly less deeply into the GCL at all time-points analyzed; a small fraction even migrates in the opposite direction (negative values) towards the hilus. Note that the fraction of these cells remains constant over time, indicating that they survive for at least 42 days. For statistical comparison, Kolmogorov-Smirnov test was used for pair-wise comparisons of the distribution curves (**** $P < 0.0001$ compared to wild type and $\alpha 5$ -KO eGFP-positive GCs). Panel D also illustrates the migration pattern of GCs over-expressing Cdk5 in wild type and $\alpha 5$ -het mice, demonstrating phenotypic rescue in the mutant (Kolmogorov-Smirnov test: **** $P < 0.0001$ compared to eGFP-positive GCs in $\alpha 5$ -het mice). **F–H**. Cumulative distribution analysis of the migration distance in the GCL of mRFP-positive (wild type) and mRFP/eGFP-positive ($\alpha 5$ -KO cond.) GCs at 14 (F), 28 (G) and 42 (H) dpi. At all three time-points, a significantly reduced migrated distance was measured in double-

positive GCs (Kolmogorov-Smirnov test: $P < 0.0001$). Abbreviations: GCL, granule cell layer; ML, molecular layer; SGZ, subgranular zone. Scale bar 10 μm .

Figure 2. Dendritic development of eGFP-positive newborn neurons in wild type, $\alpha 5$ -KO and $\alpha 5$ -het mice. **A.** Representative images of dendritic arborization at 28 and 42 dpi of eGFP-positive newborn GCs, illustrating the reduced complexity in $\alpha 5$ -het mice. The dotted lines indicate the borders of the GCL; ml = molecular layer of the DG. **B–E.** Quantification of dendritic arborization by Sholl analysis at 7, 14, 28 and 42 dpi. The number of intersections between eGFP-positive dendrite segments and virtual concentric lines centered on the cell body and spaced by 10 μm is depicted (mean SEM; $n = 3$ –6 mice/group). For statistical comparison between groups, the area-under-the-curve was compared by one-way ANOVA at each time-point, followed by Tukey *post-hoc* test ($P < 0.05$). No difference in dendritic complexity was observed between wild type and $\alpha 5$ -KO mice. Newborn neurons in $\alpha 5$ -het mice had normal

growth of dendrites up to 7 dpi, but became significantly different at 14 ($*P=0.01$), 28 ($***P=0.007$) and 42 dpi ($***P=0.0011$). Panel D also illustrates the effect of Cdk5 overexpression for 28 days in $\alpha 5$ -het and wild type mice, showing diminished area-under-the curve for the latter and a trend towards an increase for the former ($***P=0.001$ compared to $\alpha 5$ -KO). **F–H.** Quantification of proximal dendritic length, total dendritic length and number of terminal branches, represented by bar-graphs (mean SEM; $N=3$ –6 mice per group). No significant difference was observed between wild type and $\alpha 5$ -KO mice for all morphological parameters $***P<0.001$, compared to eGFP-positive wild type GCs; Bonferroni *post-hoc* tests). Newborn GCs of $\alpha 5$ -het mice showed a significant decrease in total dendritic length at 28 and 42 dpi, and a decrease in terminal branches at 28 dpi. Each panel also depicts in the 28 dpi group the effect of Cdk5 overexpression. Note the rescue of the number of terminal branches in $\alpha 5$ -het-Cdk5 mice ($*P<0.05$; Tukey *post-hoc* tests). Scale bar 20 μm .

Figure 3.

Dendritic development of newborn GCs in $\alpha 5$ -floxed mice. **A, B.** Representative images showing dendritic arborization of wild type and KO cells ($\alpha 5$ -KO cond.) at 28 (A) and 42 (B) dpi; the dotted line indicate the borders of the GCL. **C–E.** Quantification of dendritic arborization by Sholl analysis at 14 (C), 28 (D) and 42 (E) dpi. The number of intersections between eGFP-positive dendrite segments and virtual concentric lines centered on the cell body and spaced by 10 μm is depicted (mean $\pm$ SEM; $n = 3$ –6 mice per group). No difference in dendritic complexity was observed at 14 dpi. At 28 and 42 dpi, mRFP/eGFP-positive GCs revealed a significant reduction in dendritic complexity at the distal dendritic part (between 40 and 200 μm away from the soma) (* $P < 0.05$; unpaired t-test). **F–H.** Analysis of dendritic morphology in wild type and KO newborn GCs. Single-cell deletion of *Gabra5* resulted in a significant reduction in total dendritic length and number of terminal

branches at 28 dpi (* $P < 0.05$; Bonferroni *post-hoc* tests). **I–K.** Analysis of dendritic morphology in 28 dpi newborn neurons of $\alpha 5^{fl/+}$ mice injected with vectors expressing either Cre-mCherry or eGFP-Cdk5. No significant effect was observed for any of the three parameters analyzed. Scale bar, 20 μm .

Figure 4.

Spine density. **A.** Representative images of spines on randomly chosen distal dendritic segments of wild type, α 5-KO and α 5-het mice at 28 and 42 dpi. **B.** Quantification of spine density (mean $\pm$ SEM; $n = 3-4$ mice per group) indicated as number of spines/ μ m. No significant difference was observed between the three genotypes at either time-point (one-way ANOVA). Scale bar 5 μ m.

Figure 5.

Changes in expression levels of different GABA_AR α subunits. **A–B.** Representative digital images depicting α1 (A–A'') and α5 (B–B'') subunit-immunoreactivity in the hippocampal formation of wild type, α5-het, and α5-KO mice. Note the increased α1 subunit staining in α5-KO mice and the unchanged α5 subunit staining in α5-het mice, compared to wild type. Panel B'' documents the complete loss of α5 subunit-immunoreactivity in α5-KO tissue. **C.** Histograms depicting changes in immunoreactivity for each subunit in 9 different brain regions of α5-het and α5-KO mice, relative to wild type (WT). Note the general trend for increased staining in α5-KO and lack of change in α5-het mice. While significant genotype and region-specific effects are seen for each subunit, post-hoc tests only confirmed the increased α1 subunit-immunoreactivity and the decreased α3 subunit staining in the

thalamic reticular nucleus in $\alpha 5$ -KO mice (Bonferonni post-hoc tests; * $P < 0.05$; *** $P < 0.001$; $n = 5/\text{genotype}$) compared to wild type mice. Scale bar = 0.2 mm.

Table 1

List of primary antibodies

Target protein	Species	Dilution	Source; catalog	Method
GFP	chicken	1:2000	Aves Laboratories; GFP-1020	IF
mRFP	rat	1:2000	Chromo Tek; RFP 5F8	IF
GABA _A R α 1-subunit	Guinea pig	1:30'000	Self-made; Fritschy and Mohler, 1995	Peroxidase
GABA _A R α 2-subunit	Guinea pig	1:2000	Self-made; Fritschy and Mohler, 1995	Peroxidase
GABA _A R α 3-subunit	Guinea pig	1:12'000	Self-made; Fritschy and Mohler, 1995	Peroxidase
GABA _A R α 4-subunit	rabbit	1:2000	PhosphoSolutions, Aurora	Peroxidase
GABA _A R α 5-subunit	Guinea pig	1:12'000	Self-made; Fritschy and Mohler, 1995	Peroxidase

IF, immunofluorescence

Table 2Densitometric analysis of GABA_A receptor subunit immunoreactivity

	mean ± SD	mean ± SD	mean ± SD
α1 subunit	wild type	α5-het	α5-KO
CA1	0.63 ± 0.03	0.64 ± 0.08	<i>0.88 ± 0.14</i>
CA3	0.58 ± 0.04	0.57 ± 0.09	0.71 ± 0.10
DG	0.65 ± 0.03	0.63 ± 0.04	<i>0.92 ± 0.15</i>
Hilus	0.44 ± 0.03	0.48 ± 0.03	<i>0.63 ± 0.07</i>
VB	0.79 ± 0.08	0.71 ± 0.12	0.81 ± 0.08
CPu	0.60 ± 0.07	0.50 ± 0.04	0.62 ± 0.05
Ctx2–3	0.74 ± 0.06	0.70 ± 0.10	<i>0.92 ± 0.08</i>
Ctx4	0.82 ± 0.04	0.81 ± 0.06	<i>1.00 ± 0.11</i>
Ctx5–6	0.64 ± 0.03	0.61 ± 0.10	<i>0.82 ± 0.11</i>
α2 subunit			
CA1	0.61 ± 0.04	0.69 ± 0.06	0.77 ± 0.08
CA3	0.85 ± 0.06	0.90 ± 0.07	1.00 ± 0.14
DG	0.86 ± 0.07	0.99 ± 0.07	0.97 ± 0.10
Hilus	0.34 ± 0.03	0.36 ± 0.07	0.31 ± 0.13
VB	0.46 ± 0.05	0.36 ± 0.08	0.34 ± 0.05
CPu	0.57 ± 0.18	0.66 ± 0.03	0.72 ± 0.06
Ctx2–3	0.31 ± 0.06	0.37 ± 0.09	0.38 ± 0.08
Ctx4	0.23 ± 0.05	0.28 ± 0.07	0.28 ± 0.06
Ctx5–6	0.21 ± 0.05	0.26 ± 0.07	0.25 ± 0.04
α3 subunit			
CA1	0.51 ± 0.07	0.45 ± 0.03	0.64 ± 0.03
CA3	0.50 ± 0.09	0.41 ± 0.06	0.56 ± 0.05
DG	0.38 ± 0.07	0.26 ± 0.04	0.37 ± 0.05
Hilus	0.29 ± 0.06	0.25 ± 0.03	0.34 ± 0.03
NRT	1.00 ± 0.06	<i>0.74 ± 0.10</i>	0.96 ± 0.10
CPu	0.64 ± 0.16	0.55 ± 0.04	0.68 ± 0.08
Ctx2–3	0.40 ± 0.08	0.38 ± 0.09	0.48 ± 0.07
Ctx4	0.29 ± 0.08	0.29 ± 0.07	0.32 ± 0.08
Ctx5–6	0.46 ± 0.07	0.45 ± 0.08	0.55 ± 0.09
α4 subunit			
CA1	0.66 ± 0.54	0.62 ± 0.44	0.87 ± 0.64
CA3	0.68 ± 0.51	0.58 ± 0.39	0.86 ± 0.46
DG	0.54 ± 0.35	0.41 ± 0.21	0.57 ± 0.30
Hilus	0.39 ± 0.30	0.39 ± 0.20	0.59 ± 0.23
VB	0.72 ± 0.57	0.53 ± 0.31	0.62 ± 0.47
CPu	1.00 ± 0.50	0.69 ± 0.61	0.89 ± 0.72
Ctx2–3	0.55 ± 0.38	0.57 ± 0.33	0.68 ± 0.45
Ctx4	0.42 ± 0.27	0.44 ± 0.23	0.46 ± 0.30

	mean $\pm$ SD	mean $\pm$ SD	mean $\pm$ SD
$\alpha 1$ subunit	wild type	$\alpha 5$-het	$\alpha 5$-KO
Ctx5–6	0.62 $\pm$ 0.47	0.64 $\pm$ 0.43	0.70 $\pm$ 0.59
$\alpha 5$ subunit			
CA1	0.81 $\pm$ 0.08	0.93 $\pm$ 0.08	
CA3	0.91 $\pm$ 0.12	1.00 $\pm$ 0.15	
DG	0.61 $\pm$ 0.10	0.74 $\pm$ 0.08	
Hilus	0.36 $\pm$ 0.11	0.38 $\pm$ 0.08	
VB	0.30 $\pm$ 0.04	0.27 $\pm$ 0.04	
CPu	0.26 $\pm$ 0.17	0.33 $\pm$ 0.06	
Ctx2–3	0.22 $\pm$ 0.04	0.27 $\pm$ 0.07	
Ctx4	0.20 $\pm$ 0.04	0.23 $\pm$ 0.06	
Ctx5–6	0.32 $\pm$ 0.05	0.38 $\pm$ 0.06	

All optical density values were calibrated using a gray intensity scale and normalized for each subunit to the highest value read in the sample. Italics indicate statistically significant changes (Two-way ANOVA; Bonferroni post-hoc test; $P < 0.05$; $n = 5$ mice/genotype). Abbreviations: CPu: striatum; Ctx2–3: layers 2–3; Ctx 4: layer 4; Ctx5–6: layers 5–6 of somatosensory cortex; DG: dentate gyrus molecular layer; NRT: thalamic reticular nucleus; VB: thalamic ventrobasal complex