RESEARCH ARTICLE

GABA suppresses neurogenesis in the adult hippocampus through GABA_B receptors

Claudio Giachino1, Michael Barz2, Jan S. Tchorz3, Mercedes Tome3, Martin Gassmann3, Josef Bischofberger2, Bernhard Bettler3 and Verdon Taylor1,*

ABSTRACT

Adult neurogenesis is tightly regulated through the interaction of neural stem/progenitor cells (NSCs) with their niche. Neurotransmitters, including GABA activation of GABA_A receptor ion channels, are important niche signals. We show that adult mouse hippocampal NSCs and their progeny express metabotropic GABA_B receptors. Pharmacological inhibition of GABA_B receptors stimulated NSC proliferation and genetic deletion of GABA_B1 receptor subunits increased NSC proliferation and differentiation of neuroblasts in vivo. Cell-specific conditional deletion of GABA_B receptors supports a cell-autonomous role in newly generated cells. Our data indicate that signaling through GABA_B receptors is an inhibitor of adult neurogenesis.

KEY WORDS: GABA_B receptors, Neurotransmitters, Neural stem cells, Mouse

INTRODUCTION

The adult hippocampus contains neural stem/progenitor cells (NSCs) within a specialized subgranular zone (SGZ) niche of the dentate gyrus (DG) (Kempermann et al., 2004). Hippocampal NSCs depend on canonical Notch signaling for their maintenance and express the Notch target gene Hes5 (Breunig et al., 2007; Ables et al., 2010; Ehmann et al., 2010; Lugert et al., 2010). Hes5+ NSCs produce intermediate progenitors that generate proliferating neuroblasts, which exit the cell cycle before differentiating into granule neurons (Lugert et al., 2012). Neurogenesis is tightly regulated through a balance of NSC maintenance and differentiation signals within the SGZ niche. Neurotransmitters may mediate crosstalk between newly generated cells and the surrounding neuronal network (Masiulis et al., 2011). Under physiological conditions, DG neurogenesis is modulated by neural excitation (Deisseroth et al., 2004; Tozuka et al., 2005; Parent, 2007) and accumulating evidence indicates that neurotransmitters can influence the proliferation and differentiation of newborn cells (Ge et al., 2006; Jagasia et al., 2009; Jhaeveri et al., 2010; Duveau et al., 2011; Song et al., 2012). GABA is the major inhibitory neurotransmitter in the adult brain acting via two main receptor types: ionotropic GABA_A and G-protein coupled metabotropic GABA_B receptors. Adult neurogenesis is sensitive to GABA_A receptor signaling (Masiulis et al., 2011; Song et al., 2012); however, a role for GABA signaling through GABA_B receptors in the regulation of adult NSCs remains poorly defined.

GABA_B receptors are heterodimers composed of GABA_B1 and GABA_B2 (Gabbr1 and Gabbr2 – Mouse Genome Informatics) subunits, both of which are required for normal receptor function (Ulrich and Bettler, 2007). Accordingly, mice lacking the GABA_B1 subunit (Gabbr1−/−; hereafter GABA_B1−/−) show a complete absence of GABA_B responses (Schuler et al., 2001). Distinct isoforms of GABA_B1 receptor subunits (GABA_B1a and GABA_B1b) are generated from the GABA_B1 gene by differential promoter usage. Receptors containing GABA_B1a and GABA_B1b subunits exhibit a preferential axonal versus dendritic distribution, respectively, and accordingly they mediate distinct synaptic functions (Pérez-Garcia et al., 2006; Vigot et al., 2006). GABA_B receptors regulate neuronal excitability controlling the activity of voltage-gated calcium channels and inward-rectifying potassium channels (Ulrich and Bettler, 2007). GABA_B receptors affect progenitor proliferation and migration in the developing brain (Fukuoka et al., 2008; Salazar et al., 2008; Wang and Kriegstein, 2009). However, whether GABA_B receptors play a role in adult NSC biology in vivo is unclear.

Here we employed genetic and pharmacological approaches to investigate GABA_B receptor function in regulating adult hippocampal neurogenesis. We show that GABA_B receptors are expressed by many cell types in the adult DG. GABA_B signaling is active in cells throughout the adult neurogenic lineage including the most primitive Hes5-expressing quiescent NSCs. Genetic and pharmacological inhibition of GABA_B receptor signaling increases proliferation of Hes5+ NSCs, and increases the production of new neurons. Hence, our data indicate that GABA_B signaling is an important inhibitor of adult neurogenesis and promotes the quiescence of NSCs in the DG though an ion-channel-independent mechanism.

RESULTS

GABA_B receptors are expressed by cells in the adult neurogenic niche

GABA_B receptors are expressed by most hippocampal neurons in mice (Fig. 1A-F) (Schuler et al., 2001); however, it is not known whether they are expressed by newly generated cells in the SGZ. To address whether newly generated adult granule neurons express GABA_B receptors, we labeled proliferating cells in vivo with bromodeoxyuridine (BrdU) followed by a chase period of 30 days to allow for maturation of BrdU-labeled cells. Most BrdU-labeled neurons expressed GABA_B1 and GABA_B2 subunits (Fig. 1G,H) suggesting that GABA_B signaling may have cell-autonomous functions in adult-generated granule cells. We also observed GABA_B1-expressing NeuN-negative cells in the SGZ (Fig. 1F). We analyzed mice expressing functional GABA_B1-GFP fusion proteins under the control of GABA_B1 regulatory elements (Fig. 1I) (Casanova et al., 2009). GABA_B1-GFP colocalized with the neuroblast marker polysialylated neural cell adhesion molecule and brain lipid binding protein in progenitor cells (Fig. 1J,K). Hence,
GABA<sub>B1</sub> receptors were expressed by cells early within the neurogenic lineage and before neuronal maturation. The Notch target Hes5 is expressed by NSCs in the adult DG segregating the most primitive Sox2<sup>+</sup> progenitors from more committed cells (Lugert et al., 2010; Lugert et al., 2012). By analyzing Hes5::GFP mice we found that Hes5<sup>+</sup> NSCs expressed GABA<sub>B1</sub> and GABA<sub>B2</sub> subunits (radial, 86%; horizontal, 76%). These data indicate that adult hippocampal NSCs and their progeny express GABA<sub>B</sub> receptors.

Increased adult progenitor proliferation in GABA<sub>B1<sup>−/−</sup></sub> mice

We addressed whether GABA<sub>B1</sub> receptors play a role in adult hippocampal neurogenesis by analyzing GABA<sub>B1<sup>−/−</sup></sub> mice (Schuler et al., 2001). The number of proliferating [proliferating cell nuclear antigen (PCNA) or phospho-histone-H3-expressing] cells in the SGZ and granule cell layer (GrL) in GABA<sub>B1<sup>−/−</sup></sub> mice was significantly increased compared with wild-type controls (Fig. 2A-C; supplementary material Fig. S1A-C). PCNA<sup>+</sup> cells in the adult DG include partially overlapping Sox2 progenitor and Doublecortin<sup>+</sup> (Dcx) neuroblast populations (Fig. 2D-G) (Kempermann et al., 2004; Lugert et al., 2010). PCNA<sup>+</sup> Sox2<sup>−</sup> Dcx<sup>−</sup> progenitors but not PCNA<sup>+</sup> Dcx<sup>+</sup> neuroblasts were increased in GABA<sub>B1<sup>−/−</sup></sub> mice, indicating that enhanced proliferation results from activation of the more undifferentiated progenitor populations (Fig. 2H). Moreover, the number of Dcx-expressing neuroblasts was increased, whereas Sox2<sup>+</sup> progenitors were slightly decreased per mm<sup>2</sup> in the DG of GABA<sub>B1<sup>−/−</sup></sub> mice, suggesting augmented neurogenesis and enhanced differentiation.
progenitor differentiation (Fig. 2I). Taken together, these results indicate that GABA_B receptor activity controls the number of proliferating progenitors in the adult hippocampus.

**Accelerated neuronal differentiation in GABA_B1−/− mice**

We followed the differentiation of newborn cells in GABA_B1−/− mice (Fig. 3A). Two weeks after BrdU labeling, the number of newly generated cells was four times higher in GABA_B1−/− mice than in control littersmates, consistent with the increased proliferation seen in the SGZ (Fig. 3B,C,F). At this time point after BrdU labeling, neuronal differentiation of BrdU+ cells was apparent, with overlapping expression of Dcx and NeuN (Fig. 3D,E) (Kempermann et al., 2004). The proportion of newly generated BrdU+ NeuN+ mature granule cells was significantly increased in GABA_B1−/− mice at the expense of BrdU+ neuroblasts and Dcx/NeuN double-positive immature neurons (Fig. 3G). Neurogenesis and differentiation were also enhanced in the GABA_B1−/− mice after a 30-day chase (supplementary material Fig. S2A-E). Therefore, accelerated neuronal maturation, in addition to increased cell proliferation, contributes to enhanced neurogenesis in the DG of adult GABA_B1−/− mice.

**Unaltered cell survival in GABA_B1−/− mice**

We addressed whether enhanced survival, in addition to augmented proliferation and differentiation, is responsible for the increased number of newly generated granule neurons observed in GABA_B1−/− mice. To analyze apoptosis, we performed TUNEL assays in control (Fig. 4A) and GABA_B1−/− mice and quantified TUNEL-labeled cells that incorporated BrdU was increased in GABA_B1 conditional knockouts compared with controls, suggesting that loss of GABA_B1 receptors induces cell proliferation (Fig. 5B-D). The proportion of rGFP+ cells that incorporated BrdU was increased in GABA_B1 conditional knockouts compared with controls, suggesting that loss of GABA_B1 receptors induces cell proliferation (Fig. 5B-D). The proportion of rGFP+ cells that expressed neuronal markers (Dcx or NeuN) and incorporated BrdU was also increased, indicating enhanced neurogenesis (Fig. 5E).

**GABA_A receptor antagonist activates quiescent NSCs, whereas GABA_B receptor agonist promotes NSC quiescence**

Most adult hippocampal NSCs are quiescent (Kronenberg et al., 2003; Lugert et al., 2010; Bonaguidi et al., 2011; Dranovsky et al., 2011). NSC quiescence is reversible in response to a number of pathophysiological stimuli (Lugert et al., 2010; Bonaguidi et al., 2011; Dranovsky et al., 2011). Neurotransmitters can directly regulate hippocampal NSC quiescence (Jhaveri et al., 2010; Song et al., 2012). The increased progenitor proliferation in GABA_B1−/− mice suggested that GABA_B receptors may modulate NSCs quiescence. We inhibited GABA_A receptor function by infusing the GABA_A antagonist CGP54626A (CGP) intracranially for six consecutive days into Hes5::GFP mice (Fig. 6A). Proliferation (PCNA− cells) increased dramatically in the SGZ of CGP- versus saline-treated mice (Fig. 6B,C,F) and the density of Hes5− PCNA− cells was increased, indicating that Hes5− NSCs were affected (Fig. 6D,E,G).
Interestingly, although the proportion of Hes5::GFP\(^+\) cells that expressed PCNA increased after CGP infusion, the density of Hes5::GFP\(^+\) cells was unchanged (Fig. 6H,I). This implied that although blocking GABA\(_B\) function recruited quiescent cells to the active proliferative stem cell pool it did not induce an expansion of the stem cell population.

In a complementary approach, we activated GABA\(_B\) receptors by intracranial infusion of the GABA\(_B\) agonist baclofen for six consecutive days into Hes5::GFP mice (supplementary material Fig. S3A). The density of PCNA\(^+\) cells and Hes5\(^+\) cells did not decrease significantly in the SGZ of baclofen- versus saline-treated mice (supplementary material Fig. S3B-F). However, the proportion of Hes5::GFP\(^+\) cells that expressed PCNA decreased after baclofen infusion, suggesting that Hes5\(^+\) NSCs were preferentially affected and switched to a quiescent state (supplementary material Fig. S3G).

**DISCUSSION**

Much effort has been put into understanding the regulation of neurogenesis in the hippocampus of adult mammals and the functions of these newborn neurons in homeostasis and disease. However, our knowledge of how the brain coordinates network activity and the generation of new neurons is still limited. GABA released by local interneurons is a major extrinsic regulator that can profoundly affect adult hippocampal neurogenesis (Masiulis et al., 2011; Song et al., 2012). The action of GABA on neural stem and progenitor cell proliferation is complex and still controversial.

GABA can promote or suppress proliferation depending on developmental stage, brain region and the fate of distinct progenitor populations (Haydar et al., 2000; Liu et al., 2005; Duveau et al., 2011). In the adult hippocampus, ionotropic GAB\(_A\) receptors have been reported to decrease cell proliferation (Duveau et al., 2011; Song et al., 2012). It remains unclear whether differential regulation occurs at the level of intermediate progenitors and neuroblasts (Tozuka et al., 2005; Ge et al., 2006) versus NSCs (Wang et al., 2005; Song et al., 2012). Moreover, although ionotropic GAB\(_A\) receptors mediate most of the GABA effects on adult neurogenesis described to date, little is known of the function of GABA\(_B\) receptors in this context (Felice et al., 2012). We provide evidence that metabotropic GABA\(_B\) receptors may directly suppress NSC proliferation and neuroblast differentiation in the adult hippocampus.

Our results show that GABA signaling through GABA\(_B\) receptors inhibits DG NSC proliferation. We propose that this inhibition is, at least in part, a direct effect of GABA\(_B\) signaling in NSCs. Neurotransmitters may mediate crosstalk between newly generated cells and the surrounding neuronal network, thereby matching neural activity with neurogenic output (Masulis et al., 2011). Signaling via GABA\(_B\) receptors is a novel regulator that may contribute to coordinate hippocampal network activity and NSC proliferation. Understanding the molecular mechanisms regulating proliferation versus quiescence of adult NSCs is crucial. NSCs become mostly quiescent during aging, and this correlates with a dramatic reduction in neurogenesis with age (Hattiangady and Shetty, 2008; Jessberger et al., 2011).
and Gage, 2008; Lugert et al., 2010). However, NSC quiescence is reversible, and this could be exploited to rejuvenate neurogenesis in the aged or damaged brain (Hattiangady and Shetty, 2008; Lugert et al., 2010). Importantly, excitation as well as specific neurotransmitters can activate the latent stem cell pool (Jhaveri et al., 2010; Lugert et al., 2010), and here we propose that GABAB receptors can contribute to this process. Therefore, manipulation of GABAB function may be a novel approach to modulate adult hippocampal neurogenesis in vivo and during aging. Recently, GABAB receptors have attracted attention as potentially being involved in the etiology of depression, and GABAB blockade causes antidepressant-like effects (Cryan and Slattery, 2010). Given that antidepressant drugs can promote adult neurogenesis and new hippocampal neurons have been implicated in mediating some effects of antidepressants (Petrik et al., 2012), our findings are relevant for human disease. Indeed, increased proliferation in the

Fig. 5. GABA<sub>B</sub> deficiency cell-autonomously affects adult neurogenesis. (A) Tamoxifen (TAM) and BrdU induction regimes in GFAP-CreER<sup>T2</sup>, rGFP and GABA<sub>B<sub>1</sub></sup><sub>lox511/lox511</sub> conditional knockout (cKO) mice or GABA<sub>B<sub>1</sub></sup><sub>lox511</sub><sub>/+</sub> control mice. TAM was injected once per day for five consecutive days before the mice were sacrificed (<dollar>†</dollar>) 35 days after the end of induction. BrdU was administered through the drinking water for 7 days starting from 2 weeks after the end of TAM induction to detect early changes after conditional deletion. (B,C) Conditional GABA<sub>B<sub>1</sub></sub> loss promotes proliferation (BrdU incorporation) in comparison to control mice. The majority of the BrdU<sup>-</sup> rGFP<sup>+</sup> cells acquired a neuronal phenotype (Dcx<sup>+</sup> and/or NeuN<sup>+</sup>) 2 weeks after BrdU administration. (D) BrdU<sup>-</sup> rGFP<sup>+</sup> cells are significantly increased in GABA<sub>B<sub>1</sub></sub> conditional mutants (control 6.5±1.5; cKO 12±1.3; <i>n</i>=7/6). (E) The proportion of BrdU-labeled cells among rGFP<sup>+</sup> neuronal cells (Dcx<sup>+</sup> and NeuN<sup>+</sup>) also increases in GABA<sub>B<sub>1</sub></sub> cKO mice compared with controls (control 13±1.15; cKO 19.7±0.88; <i>n</i>=3). <i>t</i>-test: <i>**P</i>&lt;0.05. Error bars indicate s.e.m. Scale bars: 10 μm.

Fig. 6. Infusion of GABA<sub>B</sub> antagonist activates adult hippocampal NSCs. (A) GABA<sub>B</sub> antagonist induction regime. CGP54626A (CGP) was infused for 6 days into the hippocampus of adult Hes5::GFP<sup>+</sup> mice. The mice were sacrificed (<dollar>†</dollar>) at day 6 (<i>d6</i>). (B,C) Representative images of proliferating cells (PCNA<sup>+</sup>), neuroblasts (Dcx<sup>+</sup>) and NSCs (Hes5::GFP<sup>+</sup>) in the SGZ of CGP and control (saline) infused mice. (D,E) CGP induces Hes5::GFP<sup>+</sup> cells to proliferate (arrows). (F) The density of PCNA<sup>+</sup> proliferating cells is increased in CGP-treated mice (control 456±108; CGP 1268±299; <i>n</i>=5/7). (G) Proliferating (PCNA<sup>+</sup>) Hes5::GFP<sup>+</sup> cells are increased in number in CGP-treated mice (control 94±8; CGP 195±32; <i>n</i>=5/7). (H) The Hes5::GFP<sup>+</sup> population does not expand after CGP treatment (control 1267±74; CGP 1489±69; <i>n</i>=5/7; <i>P</i>=0.06). (I) The proportion of Hes5::GFP<sup>+</sup> cells that proliferate (PCNA<sup>+</sup>) increases after CGP treatment (control 7.4±0.3; CGP 12.8±1.8; <i>n</i>=5/7). <i>t</i>-test: <i>**P</i>&lt;0.05. Error bars indicate s.e.m. Scale bars: B,C, 50 μm; D,E, 10 μm.
ventral hippocampus has been suggested as a plausible mechanism for the antidepressant-like effects of chronic treatment with GABA\textsubscript{B} receptor antagonists (Felice et al., 2012).

Together, our data suggest that metabotropic GABA\textsubscript{B} receptors are already active in the cells at the start of the adult neurogenic lineage. This may represent a novel mechanism to integrate hippocampal network activity, GABA release and NSC proliferation. Based on continued expression of GABA\textsubscript{B} subunits in more differentiated cell types, further regulation by GABA\textsubscript{B} may occur downstream of NSCs during adult hippocampal neurogenesis. Indeed, our results show that differentiation of neuroblasts is accelerated in mice lacking the GABA\textsubscript{B} receptor subunits, without there being a significant effect on newborn neuron survival. Notably, and in contrast to the action of the GABA\textsubscript{A} receptors, activation of GABA\textsubscript{B} receptors promotes differentiation along the neuronal lineage, survival of new neurons as well as asynaptic integration in the adult DG (Tozuka et al., 2005; Ge et al., 2006; Jagasia et al., 2009). Therefore, GABA\textsubscript{B} receptors can potentially synergize with GABA\textsubscript{A} receptors to inhibit NSC division (Song et al., 2012) and counteract the differentiation-promoting effects of GABA\textsubscript{A} receptors later within the neurogenic lineage (Tozuka et al., 2005; Ge et al., 2006).

Little is known about the molecular mechanisms and signaling pathways that mediate the effects of neurotransmitters on adult NSCs and their progeny. GABA\textsubscript{B} receptors can modulate ion channels opening at the plasma membrane (Ulrich and Betterl, 2007). Postsynaptic GABA\textsubscript{B}R-containing receptors activate K\textsuperscript{+} channels. In contrast to the K\textsuperscript{+}-current effects of GABA\textsubscript{A} receptors on neurons, hippocampal NSCs showed leaky membrane currents and their K\textsuperscript{+}-currents were not dramatically affected by pharmacological activation of GABA\textsubscript{B} receptors (data not shown) (Filippov et al., 2003). Thus, we suggest that GABA\textsubscript{B}-induced hyperpolarization is unlikely to be the main mechanism that mediates the inhibitory action of GABA\textsubscript{B} receptors on progenitor proliferation, but this will require closer scrutiny in the future. In addition to modulating ion channels, GABA\textsubscript{B} receptors can inhibit adenylate-cyclase activity (Kaumann et al., 1997; Kuner et al., 1999; Martin et al., 1999). Activation of Beta3-adrenergic receptors, which positively regulate the adenylate cyclase via G-protein coupling and are specifically expressed by Hes\textsuperscript{5} NSCs in the SGZ, induces cell proliferation in the adult DG (Ursino et al., 2009; Jhaveri et al., 2010). The adenylate-cyclic-AMP-CREB axis is also a key signal transduction pathway that promotes neuronal differentiation in the DG (Palmer et al., 1997; Fujioka et al., 2004) and is potentiated by GABA\textsubscript{B}-mediated depolarization in SGZ neuroblasts (Jagasia et al., 2009). Thus, released inhibition of the adenylate cyclase may contribute to increased neurogenesis in the GABA\textsubscript{A}R-deficient mouse hippocampus by counteracting the effects of Beta3-adrenergic receptors and GABA\textsubscript{B} receptors in NSCs and neuroblasts, respectively. Future studies will need to address a potential role for second-messenger regulation by GABA\textsubscript{B} receptors in adult neurogenesis.

MATERIALS AND METHODS
Animals and husbandry
GABA\textsubscript{B\textsubscript{1}}, GABA\textsubscript{B\textsubscript{2}}, GABA\textsubscript{B\textsubscript{1}}, GABA\textsubscript{B\textsubscript{2}}, GABA\textsubscript{B2}, GABA\textsubscript{B2}, GABA\textsubscript{B2} have been described elsewhere (Schuler et al., 2001; Gassmann et al., 2004; Haller et al., 2004; Hirrlinger et al., 2006; Vigo et al., 2006; Casanova et al., 2009; Lugert et al., 2010; Tchores et al., 2012). Mice were maintained on a 12-hour day/night cycle with adequate food and water under specific-pathogen-free (SPF) conditions according to institutional regulations and under license numbers 35/9185.81/G-09/19 (Ethical Commission Freiburg, Germany) and 2537 and 2538 (Kantonales Veterinäramt, Basel).

BrdU and tamoxifen administration
Young adult mice (7-8 weeks old) received four consecutive intraperitoneal injections (every 2 hours) of BrdU (Sigma; 50 mg/kg body weight). Alternatively, BrdU was given to the mice for seven consecutive days dissolved in the drinking water at 0.8 mg/ml. Stock solution of tamoxifen (TAM, Sigma) were prepared at a concentration of 20 mg/ml in corn oil (Sigma). Adult mice were injected intraperitoneally with TAM once per day for five consecutive days at a dose of 2 mg per day.

CGP and baclofen infusion
Adult (2 months old) Hes5::GFP mice were anesthetized by intraperitoneal injection of a ketamine/xylazine/flunitrazepam solution (100 mg, 5 and 0.4 mg/kg body weight, respectively) and positioned in a stereotaxic apparatus (David Kopf Instruments). The skull was exposed by an incision in the scalp and a small hole (1 mm) was drilled through. Cannulas (Brain Infusion Kit 3, Alzet) were implanted at −2 mm posterior, 1.5 mm lateral to the bregma and 2 mm below the surface of the cortex to target the dorsal aspect of the anterior DG. CGP54626A (CGP, Tocris Bioscience; 500 μM in 0.9% saline), baclofen (Tocris Bioscience; 1 mM in 0.9% saline) or vehicle alone was infused for 6 days into the brain with an osmotic pump (model 1007D, Alzet). After 6 days of infusion the animals were sacrificed and analyzed. Brains were processed for immunohistochemistry as described below.

Tissue preparation, immunohistochemistry and antibodies
Mice were deeply anesthetized by injection of a ketamine/xylazine/ flunitrazepam solution (150 mg, 7.5 and 0.6 mg/kg body weight, respectively) and perfused with ice-cold 0.9% saline solution followed by ice-cold 4% paraformaldehyde (PFA) solution in 0.1 M phosphate buffer (PB). Brains were post-fixed with 4% PFA overnight, washed in PB, cryoprotected in a 30% sucrose solution in 0.1 M PB for 48 hours, frozen and sectioned at −20°C. Free-floating coronal sections (30 μm) were collected in multilevel dishes (Corning) and stored at −20°C in antifreeze solution until use.

For immunostaining, sections were incubated overnight at 4°C with the primary antibody diluted in blocking solution of 2% normal donkey serum (Jackson ImmunoResearch) 0.5% Triton X-100 in phosphate-buffered saline (PBS). Sections were washed three times in PBS and incubated at room temperature for 1 hour with the corresponding secondary antibodies in blocking solution. When necessary, sections were washed and incubated for 1 hour at room temperature in streptavidin fluorescein isothiocyanate (FITC; Jackson ImmunoResearch; 1:400). Sections were mounted on Superfrost glass slides (Thermo Scientific), embedded in mounting medium containing 1:4-diazabicyclo[2.2.2]octane (DABCO; Sigma) as an antifading agent and visualized using a Zeiss LSM510 confocal microscope. For the avidin-biotin-peroxidase method, sections were washed in PBS after incubation with secondary biotinylated antibody and then incubated for 1 hour at room temperature in peroxidase-conjugated streptavidin (Jackson ImmunoResearch; 1:1000). Sections were incubated with 0.015% 3,3'- diaminobenzidine, 0.0024% H\textsubscript{2}O\textsubscript{2} in 0.05 M Tris-HCl, pH 7.6. Sections were mounted on glass slides (Thermo Scientific), dehydrated and embedded in DePeX mounting medium (SERVA, Heidelberg, Germany).

Antibodies were used against the following antigens: NeuN (mouse, Sigma; 1:800); Calbindin D28k (mouse, Swant; 1:2000); Sox2 (rabbit, Chemicon; 1:1000); Sox2 (goat, Santa Cruz; 1:200); BLBP (rabbit, Sigma; 1:800); Calbindin D28k (mouse, Swant; 1:2000); Sox2 (rabbit, Santa Cruz; 1:1000); PCNA (rabbit, Dako; 1:1000); pH3 (rabbit, Millipore; 1:1000); Sox2 (goat, Santa Cruz; 1:200); PSA-NCAM (mouse, Chemicon; 1:2000); GABA A (rabbit 174.1; 1:300) (Malitschek et al., 1998); GABA B1 (rabbit Chemicon; 1:1500); GABA B1 (rabbit, Chemicon; 1:1000); PCNA (mouse, Dako; 1:1000); Ph3 (rabbit, Millipore; 1:100); BrdU (rat, AbD Serotec; 1:2000); Doublecortin (goat, Santa Cruz; 1:500); PSA-NCAM (mouse, Chemicon; 1:2000); GFP (sheep, Biogenesis; 1:500); PSA-NCAM (mouse, Chemicon; 1:2000); GFP (rabbit, Invitrogen; 1:500); GABA\textsubscript{B1} (mouse, Abcam; 1:300); GABA\textsubscript{B1} (rabbit 174.1; 1:300) (Malitschek et al., 1998); GABA\textsubscript{B1} (rabbit AB25; 1:1000) (Engle et al., 2006); GABA\textsubscript{B2} receptor (rabbit AB27; 1:1000); generated against a glutathione-S-transferase fusion protein containing carboxyterminal residues 746-1941 of rat GABA\textsubscript{B2} protein; Cy3/Cy5/biotin conjugated anti-mouse, rabbit, rat and goat immunoglobulins (donkey, Jackson ImmunoResearch; 1:500-1000).

Development (2014) doi:10.1242/dev.102608
TUNEL staining
Sections were washed in PBS for 10 minutes and blocked for 1 hour with 10% goat serum, 1% Trition X-100, and 0.1% bovine serum albumin (BSA) in PBS. Terminal deoxynucleotidyl transferase mediated biotinylated UTP nick end labeling (TUNEL) assays were performed according to the manufacturer’s instructions (Roche).

Quantification and statistical analyses
Immunostained hippocampal sections were analyzed on a Zeiss LSM510 confocal microscope. Data are presented as average percentages of co-labeled cells. The number of marker-positive cells in the SGZ was estimated using a 63× magnification objective. The area of the GrL was measured using ImageJ software and used to calculate the number of labeled cells per mm². Statistical comparisons were conducted by two-tailed unpaired Student’s t-test. Significance was established at P<0.05. In all graphs error bars represent standard error of the mean (s.e.m.).

Acknowledgements
We thank Dr Sebastian Lugert for comments and Frank Sager for technical assistance.

Competing interests
The authors declare no competing financial interests.

Author contributions
C.G. carried out most of the experiments and generated the figures. M.B., J.S.T., M.T. and M.G. contributed to the analysis of the GABA_B1b mutant mice, performed electrophysiology and were involved in the preparation of the manuscript. V.T., M.T. and M.G. contributed to the analysis of the GABAB mutant mice, performed confocal microscope. Data are presented as average percentages of co-labeled cells.

Funding
This work was supported by the Deutsche Forschungsgemeinschaft [DFG SFB592: TA-310-1; TA-310-2] and the Max Planck Society. We acknowledge the National Center of Competences in Research (NCCR) ‘Synapsy, Synaptic SFB592; TA-310-1; TA-310-2’ and the Max Planck Society. We acknowledge the funding from the tamoxifen-inducible DNA recombinase variant CreERT2. Glia 54, 11-20.


