RESEARCH ARTICLE

STEM CELLS AND REGENERATION

GABA suppresses neurogenesis in the adult hippocampus through GABAB receptors

Claudio Giachino[1], Michael Barz[2], Jan S. Tchorz[3], Mercedes Tome[3], Martin Gassmann[3], Josef Bischofberger[2], Bernhard Bettler[3] and Verdon Taylor[1],*

ABSTRACT

Adult neurogenesis is tightly regulated through the interaction of neural stem/progenitor cells (NSCs) with their niche. Neurotransmitters, including GABA activation of GABAA receptor ion channels, are important niche signals. We show that adult mouse hippocampal NSCs and their progeny express metabotropic GABAB receptors. Pharmacological inhibition of GABAB receptors stimulated NSC proliferation and genetic deletion of GABAB1 receptor subunits increased NSC proliferation and differentiation of neuroblasts *in vivo*. Cell-specific conditional deletion of GABAB receptors supports a cell-autonomous role in newly generated cells. Our data indicate that signaling through GABAB receptors is an inhibitor of adult neurogenesis.

KEY WORDS: GABAB 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; Ehm 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; Jhaveri 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 GABAA and G-protein coupled metabotropic GABAB receptors. Adult neurogenesis is sensitive to GABAA receptor signaling (Masiulis et al., 2011; Song et al., 2012); however, a role for GABA signaling through GABAB receptors in the regulation of adult NSCs remains poorly defined.

[1]Embryology and Stem Cell Biology, Department of Biomedicine, University of Basel, Mattenstrasse 28, CH-4058 Basel, Switzerland. [2]Department of Biomedicine, Institute of Physiology, University of Basel, Klingelbergstrasse 50/70, CH-4056 Basel, Switzerland. [3]Department of Biomedicine, Institute of Physiology, Pharmazentrum, University of Basel, Pestalozzistrasse 20, CH-4056 Basel, Switzerland.
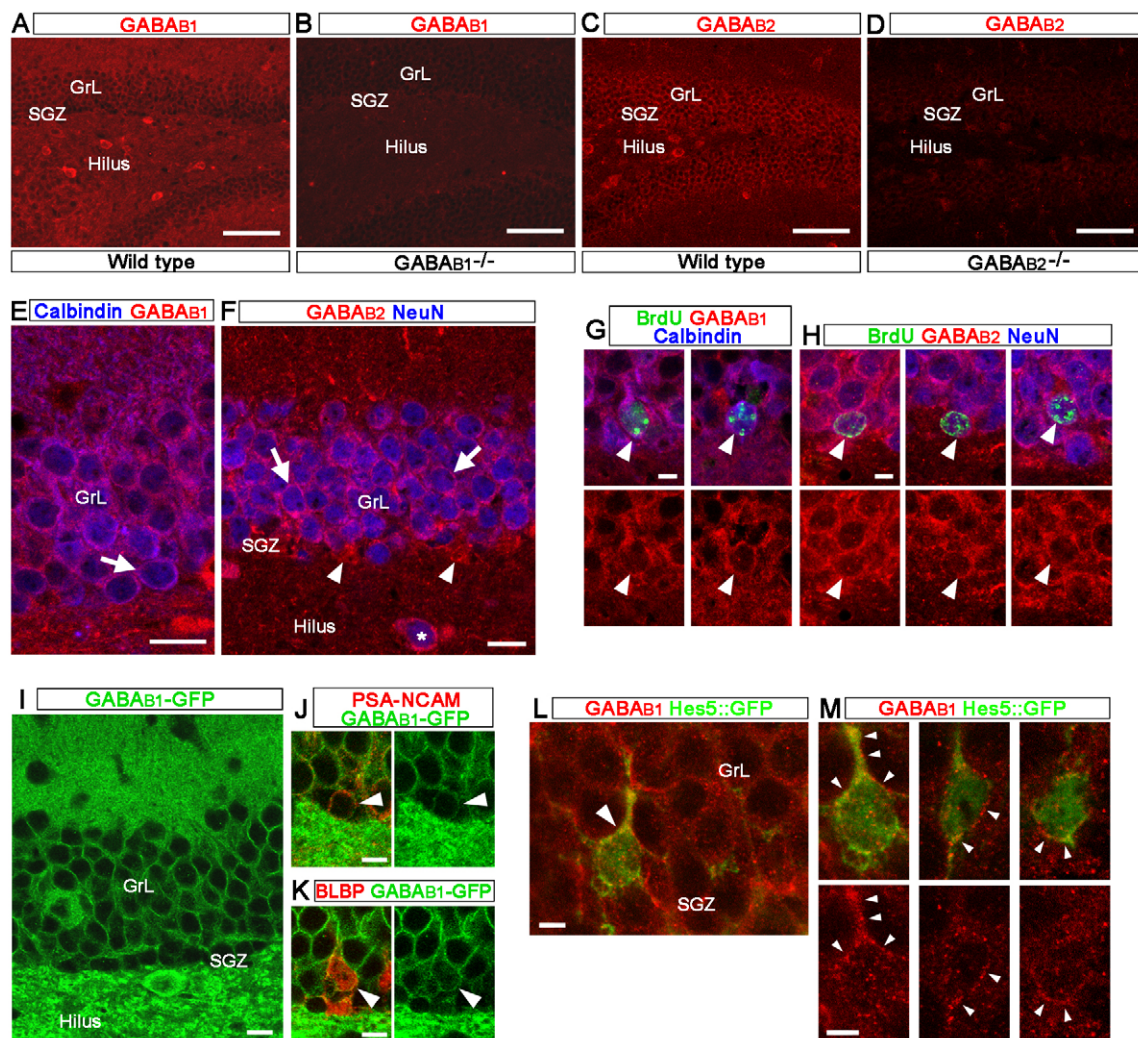
*Author for correspondence (verdon.taylor@unibas.ch)

GABAB receptors are heterodimers composed of GABAB1 and GABAB2 (Gabbr1 and Gabbr2 – Mouse Genome Informatics) subunits, both of which are required for normal receptor function (Ulrich and Bettler, 2007). Accordingly, mice lacking the GABAB1 subunit (*Gabbr1*−/−; hereafter GABAB1−/−) show a complete absence of GABAB responses (Schuler et al., 2001). Distinct isoforms of GABAB1 receptor subunits (GABAB1a and GABAB1b) are generated from the *GABAB1* gene by differential promoter usage. Receptors containing GABAB1a and GABAB1b subunits exhibit a preferential axonal versus dendritic distribution, respectively, and accordingly they mediate distinct synaptic functions (Pérez-Garci et al., 2006; Vigot et al., 2006). GABAB receptors regulate neuronal excitability controlling the activity of voltage-gated calcium channels and inward-rectifying potassium channels (Ulrich and Bettler, 2007). GABAB receptors affect progenitor proliferation and migration in the developing brain (Fukui et al., 2008; Salazar et al., 2008; Wang and Kriegstein, 2009). However, whether GABAB receptors play a role in adult NSC biology *in vivo* is unclear.

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

RESULTS

GABAB receptors are expressed by cells in the adult neurogenic niche

GABAB 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 GABAB 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 GABAB1 and GABAB2 subunits (Fig. 1G,H) suggesting that GABAB signaling may have cell-autonomous functions in adult-generated granule cells. We also observed GABAB-expressing NeuN-negative cells in the SGZ (Fig. 1F). We analyzed mice expressing functional *GABAB1-GFP* fusion proteins under the control of *GABAB1* regulatory elements (Fig. 1I) (Casanova et al., 2009). GABAB1-GFP colocalized with the neuroblast marker polysialylated neural cell adhesion molecule and brain lipid binding protein in progenitor cells (Fig. 1J,K). Hence,

Fig. 1. GABA$_B$ receptors are expressed and active in the mouse adult hippocampal neurogenic niche. (A-D) Immunostaining for GABA$_{B1}$ (A,B) and GABA$_{B2}$ (C,D) receptor subunits expression in the adult hippocampal DG of wild-type (A,C) and GABA$_{B1}^{-/-}$ (B) or GABA$_{B2}^{-/-}$ (D) mice. GABA$_{B1}$ and GABA$_{B2}$ subunits are expressed in a similar pattern in the granule cell layer (GrL), SGZ and hilus of the DG (A,C). Only weak residual background staining is visible in the mutant mice (B,D). (E,F) Most Calbindin- and NeuN-positive granule neurons express GABA$_{B1}$ and GABA$_{B2}$ subunits (arrows). Note that some NeuN-negative cells in the SGZ express GABA$_B$ receptors (F, arrowheads). The asterisk in F indicates a hilar neuron expressing GABA$_{B2}$ subunits. (G,H) Newly generated hippocampal granule neurons express GABA$_B$ receptors. Thirty days after injection of bromodeoxyuridine (BrdU), newborn neurons were identified by BrdU and Calbindin or NeuN immunostaining. BrdU-positive neurons express both GABA$_{B1}$ and GABA$_{B2}$ receptor subunits (G,H, arrowheads). (I-K) Expression of the GABA$_{B1}$-GFP transgene in the hippocampal neurogenic niche. GABA$_{B1}$-GFP fusion is expressed in the GrL, SGZ and hilus similar to the endogenous GABA$_{B1}$ subunit. PSA-NCAM positive neuroblasts (J, arrowheads) and BLBP positive progenitors (K, arrowheads) in the SGZ express GABA$_{B1}$-GFP. (L,M) Representative images showing *Hes5*::GFP expressing NSCs in the SGZ of the adult hippocampus. GABA$_{B1}$-positive cells (arrowheads) can be found among the *Hes5*-expressing population. Scale bars: A-D, 100 µm; E,F,I, 20 µm; G,H,J-M, 10 µm. GrL, granule cell layer; SGZ, subgranular zone.

GABA$_{B1}$ 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$^+$ progenitors from more committed cells (Lugert et al., 2010; Lugert et al., 2012). By analyzing *Hes5::GFP* mice we found that *Hes5*$^+$ NSCs expressed GABA$_{B1}$ subunits (Fig. 1L,M). *Hes5*$^+$ cells with both radial and horizontal morphologies expressed GABA$_{B1}$ subunits (radial, 86%; horizontal, 76%). These data indicate that adult hippocampal NSCs and their progeny express GABA$_B$ receptors.

Increased adult progenitor proliferation in GABA$_{B1}^{-/-}$ mice

We addressed whether GABA$_B$ receptors play a role in adult hippocampal neurogenesis by analyzing GABA$_{B1}^{-/-}$ 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$_{B1}^{-/-}$ mice was significantly increased compared with wild-type controls (Fig. 2A-C; supplementary material Fig. S1A-C). PCNA$^+$ cells in the adult DG include partially overlapping Sox2 progenitor and Doublecortin$^+$ (Dcx) neuroblast populations (Fig. 2D-G) (Kempermann et al., 2004; Lugert et al., 2010). PCNA$^+$ Sox2$^+$ Dcx$^-$ progenitors but not PCNA$^+$ Dcx$^+$ neuroblasts were increased in GABA$_{B1}^{-/-}$ 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$^+$ progenitors were slightly decreased per mm^2 in the DG of GABA$_{B1}^{-/-}$ mice, suggesting augmented neurogenesis and enhanced
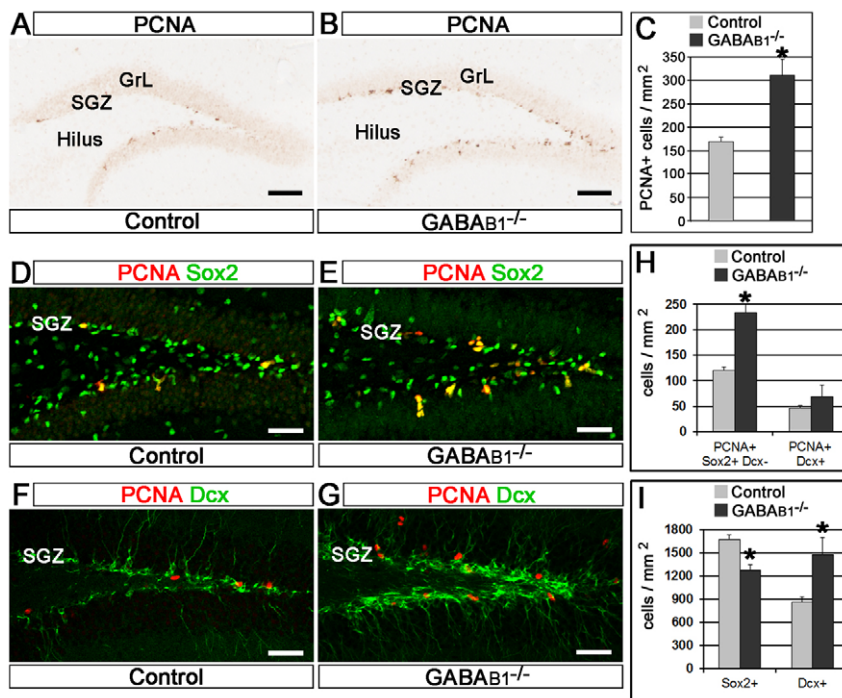
Fig. 2. Increased proliferation in the hippocampus of GABA$_{B1}$-deficient mice. (A,B) Representative images of proliferating PCNA$^+$ cells in the adult hippocampal DG of GABA$_{B1}^{-/-}$ and wild-type control mice. (C) The density of PCNA$^+$ proliferating cells is increased in mutant mice (control 169±11; GABA$_{B1}^{-/-}$ 311±33; n=6/5). (D-G) Phenotypic analysis of PCNA$^+$ proliferating cells in control (D,F) and GABA$_{B1}^{-/-}$ mice (E,G). Sox2 and Dcx were used to label progenitor cells and neuroblasts, respectively. The density of PCNA$^+$Sox2$^+$ cells (E) as well as the overall Dcx$^+$ population (G) are increased in the DG of mutant mice. (H) Most PCNA$^+$ cells express the progenitor markers Sox2 and not Dcx, and their density is increased in GABA$_{B1}$ mutants (control 120±10; GABA$_{B1}^{-/-}$ 233±40; n=6/5). The density of proliferating neuroblasts (PCNA$^+$Dcx$^+$) is unchanged in GABA$_{B1}$ mutants (control 46±6.5; GABA$_{B1}^{-/-}$ 69±13; n=6/5; P=0.16). (I) The density of Sox2$^+$ cells is slightly reduced in mutant mice (control 1671±67; GABA$_{B1}^{-/-}$ 1278±77; n=6/5) whereas that of Dcx$^+$ cells is increased (control 858±69; GABA$_{B1}^{-/-}$ 1477±220; n=6/5). t-test: *P<0.05. Error bars indicate s.e.m. Scale bars: A,B, 100 μm; D-G, 50 μm.

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 littermates, 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 GABA$_{B1}^{-/-}$ and GABA$_{B1}^{+/+}$ mice and quantified TUNEL-labeled cells in the SGZ (Fig. 4A). Loss of GABA$_{B1}$ receptor subunits did not affect apoptosis in the DG (Fig. 4B). As an independent measure of the survival of newly generated neurons, we calculated the fraction of BrdU$^+$ cells at 30 days compared to 15 days after BrdU labeling. The percentage of BrdU$^+$ cells surviving at 30 days was similar in GABA$_{B1}^{-/-}$ mice and in control littermates (Fig. 4C). Thus, increased proliferation and differentiation are the main factors responsible for the neurogenic phenotype seen in GABA$_{B1}^{-/-}$ mice,

and conversely the survival of newborn neurons is not significantly affected.

Conditional deletion of GABA$_{B1}$ subunit from adult neural progenitors affects neurogenesis

GABA$_B$ receptor subunits are expressed not only by NSCs and their progeny but also by other cells within the DG (Fig. 1). We inactivated GABA$_{B1}$ in DG NSCs using conditional floxed $GABA_{B1}^{lox511/lox511}$ and $GFAP::CreER^{T2}$ alleles (Haller et al., 2004; Hirrlinger et al., 2006) and visualized cells where Cre-recombinase had been active by following the recombination of the $mR26CS$-$EGFP$ Cre-reporter allele (rGFP) (Tchorz et al., 2012). We traced newborn cells by BrdU labeling (Fig. 5A). 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$_B$ 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$_B$ receptor function by infusing the GABA$_B$ 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).
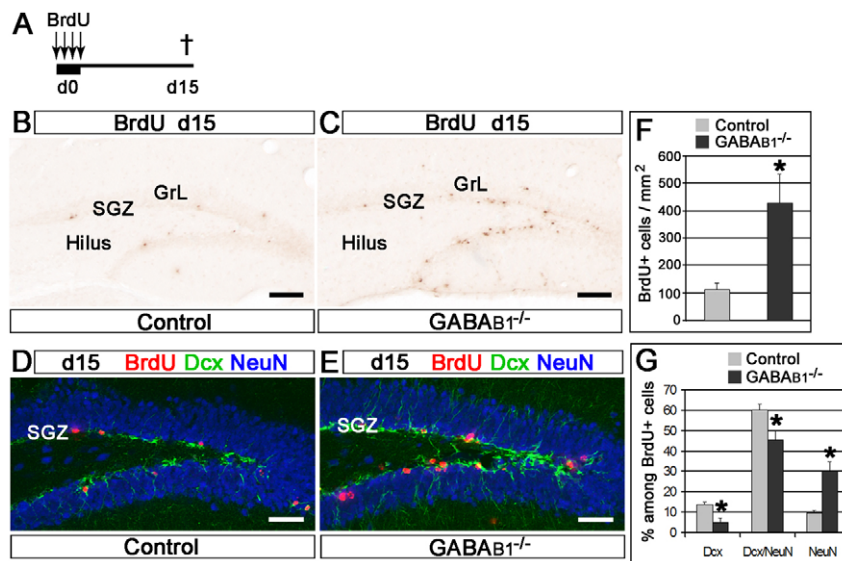
Fig. 3. Increased differentiation in the hippocampus of GABA$_{B1}$-deficient mice. (A) BrdU was injected intraperitoneally four times on day 0 (d0) to label newly generated cells and the mice were sacrificed (†) 15 days later (d15). (B,C) Representative images of BrdU$^+$ cells in the hippocampus of GABA$_{B1}$$^{-/-}$ and control mice. (D,E) Phenotypic analysis of BrdU$^+$ cells. Dcx and NeuN were used to label neuroblasts and mature neurons, respectively. (F) The density of BrdU$^+$ newborn cells is increased fourfold in mutant mice at d15 (control 111±25; GABA$_{B1}$$^{-/-}$ 429±104; n=6/5). (G) Fifteen days after BrdU injection, most BrdU$^+$ cells are differentiating neurons expressing both Dcx and NeuN. Some BrdU$^+$ cells express only Dcx (neuroblasts) and others only NeuN (mature neurons). Note that the proportion of mature neurons is increased at the expense of neuroblasts and differentiating neurons in GABA$_{B1}$ mutant mice (Dcx, control 13.6±1.3; GABA$_{B1}$$^{-/-}$ 5.2±2; Dcx/NeuN, control 60±2.7; GABA$_{B1}$$^{-/-}$ 45±4.9; NeuN, control 9.7±1.3; GABA$_{B1}$$^{-/-}$ 30±4.7; n=6/5). t-test: *P<0.05. Error bars indicate s.e.m. Scale bars: B,C, 100 μm; D,E, 50 μm.

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 GABA$_A$ 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 GABA$_A$ 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 (Masiulis 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
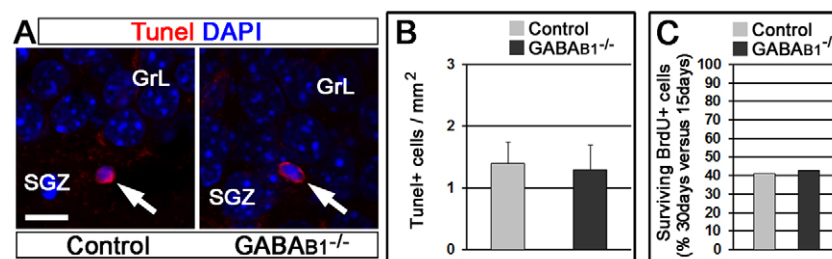

Fig. 4. Apoptosis and cell survival are not affected in the SGZ of GABA$_{B1}$-deficient mice. (A) Representative images showing pyknotic Tunel$^+$ cells in the SGZ and GrL of GABA$_{B1}$$^{-/-}$ and wild-type control mice. (B) The density of Tunel$^+$ cells in the adult hippocampus of GABA$_{B1}$$^{-/-}$ mice is unchanged compared with control mice (control 1.4±0.3; GABA$_{B1}$$^{-/-}$ 1.3±0.4; n=6). (C) Cell survival is not dramatically affected in GABA$_{B1}$$^{-/-}$ mice. Survival is depicted as proportion of BrdU$^+$ cells after a 30-day chase versus BrdU$^+$ cells after a 15-day chase (control 41.5; GABA$_{B1}$$^{-/-}$ 42.5; n=6/5). Error bars indicate s.e.m. Scale bar: 10 μm.
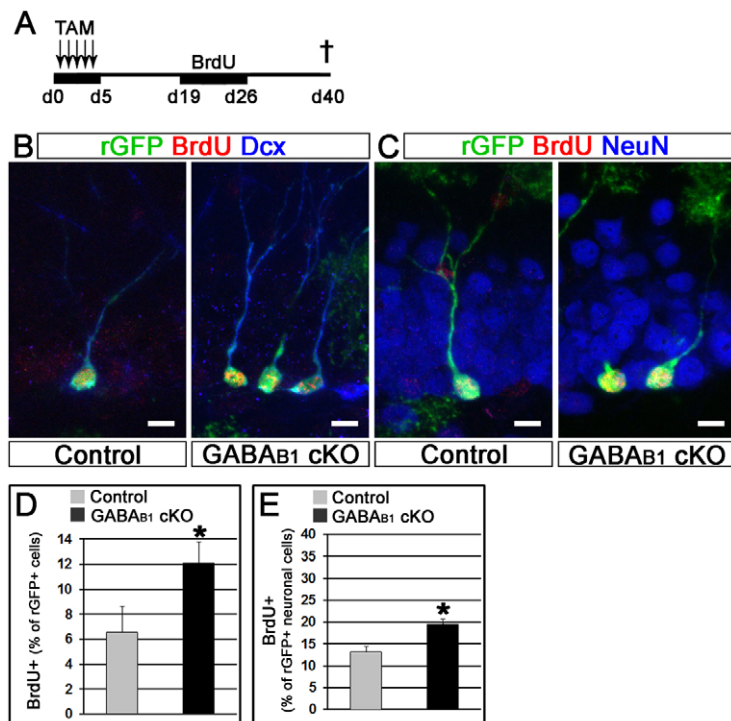
Fig. 5. GABA$_{B1}$ deficiency cell-autonomously affects adult neurogenesis. (A) Tamoxifen (TAM) and BrdU induction regimes in *GFAP::CreER^{T2}*, rGFP and GABA$_{B1}$$^{lox511/lox511}$ conditional knockout (cKO) mice or GABA$_{B1}$$^{lox511/+}$ control mice. TAM was injected once per day for five consecutive days before the mice were sacrificed (†) 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$_{B1}$ loss promotes proliferation (BrdU incorporation) in comparison to control mice. The majority of the BrdU$^+$ rGFP$^+$ cells acquired a neuronal phenotype (Dcx$^+$ and/or NeuN$^+$) 2 weeks after BrdU administration. (D) BrdU$^+$ rGFP$^+$ cells are significantly increased in GABA$_{B1}$ conditional mutants (control 6.5±1.5; cKO 12±1.3; *n*=7/6). (E) The proportion of BrdU-labeled cells among rGFP$^+$ neuronal cells (Dcx$^+$ and NeuN$^+$) also increases in GABA$_{B1}$ cKO mice compared with controls (control 13±1.15; cKO 19.7±0.88; *n*=3). *t*-test: *$P<0.05$. Error bars indicate s.e.m. Scale bars: 10 μm.

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 GABA$_B$ receptors can contribute to this process. Therefore, manipulation of GABA$_B$ function may be a novel approach to modulate adult

hippocampal neurogenesis *in vivo* and during aging. Recently, GABA$_B$ receptors have attracted attention as potentially being involved in the etiology of depression, and GABA$_B$ 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
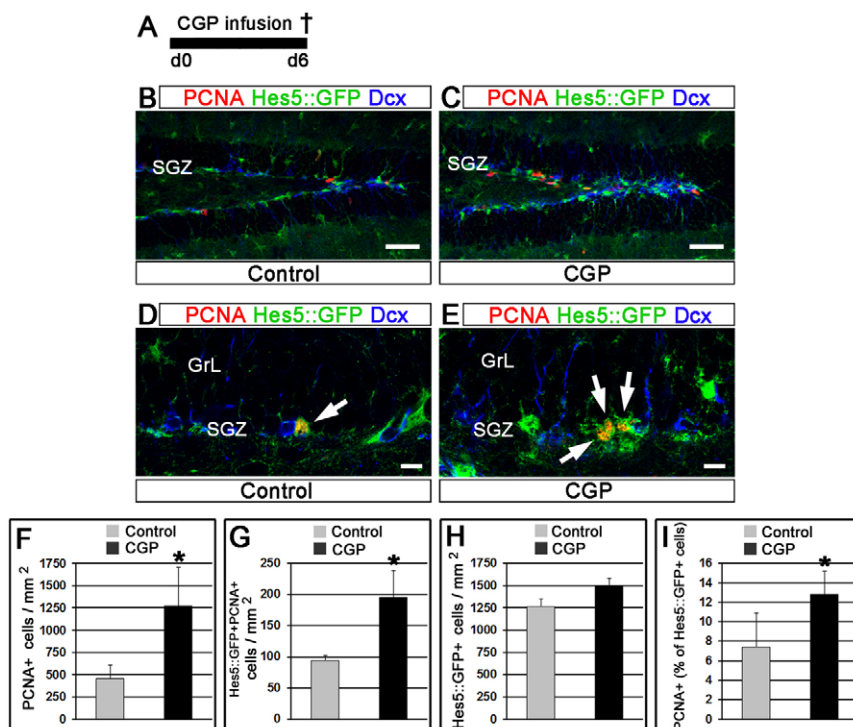

Fig. 6. Infusion of GABA$_B$ antagonist activates adult hippocampal NSCs. (A) GABA$_B$ antagonist induction regime. CGP54626A (CGP) was infused for 6 days into the hippocampus of adult *Hes5::GFP$^+$* mice. The mice were sacrificed (†) at day 6 (d6). (B,C) Representative images of proliferating cells (PCNA$^+$), neuroblasts (Dcx$^+$) and NSCs (*Hes5::GFP$^+$*) in the SGZ of CGP and control (saline) infused mice. (D,E) CGP induces *Hes5::GFP$^+$* cells to proliferate (arrows). (F) The density of PCNA$^+$ proliferating cells is increased in CGP-treated mice (control 456±108; CGP 1268±299; *n*=5/7). (G) Proliferating (PCNA$^+$) *Hes5::GFP$^+$* cells are increased in number in CGP-treated mice (control 94±8; CGP 195±32; *n*=5/7). (H) The *Hes5::GFP$^+$* population does not expand after CGP treatment (control 1267±74; CGP 1489±69; *n*=5/7; *P*=0.06). (I) The proportion of *Hes5::GFP$^+$* cells that proliferate (PCNA$^+$) increases after CGP treatment (control 7.4±0.3; CGP 12.8±1.8; *n*=5/7). *t*-test: *$P<0.05$. 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$_B$ receptor antagonists (Felice et al., 2012).

Together, our data suggest that metabotropic GABA$_B$ 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$_B$ subunits in more differentiated cell types, further regulation by GABA$_B$ may occur downstream of NSCs during adult hippocampal neurogenesis. Indeed, our results show that differentiation of neuroblasts is accelerated in mice lacking the GABA$_{B1}$ receptor subunits, without there being a significant effect on newborn neuron survival. Notably, and in contrast to the action of the GABA$_B$ receptors, activation of GABA$_A$ 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$_B$ receptors can potentially synergize with GABA$_A$ receptors to inhibit NSC division (Song et al., 2012) and counteract the differentiation-promoting effects of GABA$_A$ 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$_B$ receptors can modulate ion channels opening at the plasma membrane (Ulrich and Bettler, 2007). Postsynaptic GABA$_{B1b}$-containing receptors activate K$^+$ channels. In contrast to the K-current effects of GABA$_B$ receptors on neurons, hippocampal NSCs showed leaky membrane currents and their K-currents were not dramatically affected by pharmacological activation of GABA$_B$ receptors (data not shown) (Filippov et al., 2003). Thus, we suggest that GABA$_B$-induced hyperpolarization is unlikely to be the main mechanism that mediates the inhibitory action of GABA$_B$ receptors on progenitor proliferation, but this will require closer scrutiny in the future. In addition to modulating ion channels, GABA$_B$ receptors can inhibit adenylate-cyclase activity (Kaupmann 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 *Hes5$^+$* NSCs in the SGZ, induces cell proliferation in the adult DG (Ursino et al., 2009; Jhaveri et al., 2010). The adenylate-cyclase-cAMP-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$_A$-mediated depolarization in SGZ neuroblasts (Jagasia et al., 2009). Thus, released inhibition of the adenylate cyclase may contribute to increased neurogenesis in the GABA$_{B1}$-deficient mouse hippocampus by counteracting the effects of Beta3-adrenergic receptors and GABA$_A$ receptors in NSCs and neuroblasts, respectively. Future studies will need to address a potential role for second-messenger regulation by GABA$_B$ receptors in adult neurogenesis.

MATERIALS AND METHODS
Animals and husbandry
GABA$_{B1}$$^{-/-}$, *GABA$_{B2}$$^{-/-}$*, *GABA$_{B1}$$^{lox511/lox511}$*, *GABA$_{B1}$-GFP*, *GFAP::CreER^{T2}*, *mR26CS-EGFP* and *Hes5::GFP* mice have been described elsewhere (Schuler et al., 2001; Gassmann et al., 2004; Haller et al., 2004; Hirrlinger et al., 2006; Vigot et al., 2006; Casanova et al., 2009; Lugert et al., 2010; Tchorz 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 multiwell 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$_2$O$_2$ 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, Chemicon; 1:1500); 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); GFP (rabbit, Invitrogen; 1:500); GABA$_{B1}$ (mouse, Abcam; 1:300); GABA$_{B1}$ (rabbit 174.1; 1:300) (Malitschek et al., 1998); GABA$_{B1}$ (rabbit AB25; 1:1000) (Engle et al., 2006); GABA$_{B2}$ receptor (rabbit AB27; 1:1000; generated against a glutathione-S-transferase fusion protein containing carboxyterminal residues T746-L941 of rat GABA$_{B2}$ protein); Cy3/Cy5/biotin conjugated anti-mouse, rabbit, rat and goat immunoglobulins (donkey, Jackson ImmunoResearch; 1:500-1000).

TUNEL staining

Sections were washed in PBS for 10 minutes and blocked for 1 hour with 10% goat serum, 1% Triton 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 mm^2. 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.).

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$_B$ mutant mice, performed electrophysiology and were involved in the preparation of the manuscript. V.T., C.G., J.B. and B.B. conceived the project, designed the experiments and wrote the manuscript.

Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.102608/-/DC1

References

Ables, J. L., Decarolis, N. A., Johnson, M. A., Rivera, P. D., Gao, Z., Cooper, D. C., Radtke, F., Hsieh, J. and Eisch, A. J. (2010). Notch1 is required for maintenance of the reservoir of adult hippocampal stem cells. *J. Neurosci.* **30**, 10484-10492.

Bonaguidi, M. A., Wheeler, M. A., Shapiro, J. S., Stadel, R. P., Sun, G. J., Ming, G. L. and Song, H. (2011). In vivo clonal analysis reveals self-renewing and multipotent adult neural stem cell characteristics. *Cell* **145**, 1142-1155.

Breunig, J. J., Silbereis, J., Vaccarino, F. M., Sestan, N. and Rakic, P. (2007). Notch regulates cell fate and dendrite morphology of newborn neurons in the postnatal dentate gyrus. *Proc. Natl. Acad. Sci. USA* **104**, 20558-20563.

Casanova, E., Guetg, N., Vigot, R., Seddik, R., Julio-Pieper, M., Hyland, N. P., Cryan, J. F., Gassmann, M. and Bettler, B. (2009). A mouse model for visualization of GABA(B) receptors. *Genesis* **47**, 595-602.

Cryan, J. F. and Slattery, D. A. (2010). GABAB receptors and depression. Current status. *Adv. Pharmacol.* **58**, 427-451.

Deisseroth, K., Singla, S., Toda, H., Monje, M., Palmer, T. D. and Malenka, R. C. (2004). Excitation-neurogenesis coupling in adult neural stem/progenitor cells. *Neuron* **42**, 535-552.

Dranovsky, A., Picchini, A. M., Moadel, T., Sisti, A. C., Yamada, A., Kimura, S., Leonardo, E. D. and Hen, R. (2011). Experience dictates stem cell fate in the adult hippocampus. *Neuron* **70**, 908-923.

Duveau, V., Laustela, S., Barth, L., Gianolini, F., Vogt, K. E., Keist, R., Chandra, D., Homanics, G. E., Rudolph, U. and Fritschy, J. M. (2011). Spatiotemporal specificity of GABAA receptor-mediated regulation of adult hippocampal neurogenesis. *Eur. J. Neurosci.* **34**, 362-373.

Ehm, O., Göritz, C., Covic, M., Schäffner, I., Schwarz, T. J., Karaca, E., Kempkes, B., Kremmer, E., Pfrieger, F. W., Espinosa, L. et al. (2010). RBPJkappa-dependent signaling is essential for long-term maintenance of neural stem cells in the adult hippocampus. *J. Neurosci.* **30**, 13794-13807.

Engle, M. P., Gassman, M., Sykes, K. T., Bettler, B. and Hammond, D. L. (2006). Spinal nerve ligation does not alter the expression or function of GABA(B) receptors in spinal cord and dorsal root ganglia of the rat. *Neuroscience* **138**, 1277-1287.

Felice, D., O'Leary, O. F., Pizzo, R. C. and Cryan, J. F. (2012). Blockade of the GABA(B) receptor increases neurogenesis in the ventral but not dorsal adult hippocampus: relevance to antidepressant action. *Neuropharmacology* **63**, 1380-1388.

Filippov, V., Kronenberg, G., Pivneva, T., Reuter, K., Steiner, B., Wang, L. P., Yamaguchi, M., Kettenmann, H. and Kempermann, G. (2003). Subpopulation of nestin-expressing progenitor cells in the adult murine hippocampus shows electrophysiological and morphological characteristics of astrocytes. *Mol. Cell. Neurosci.* **23**, 373-382.

Fujioka, T., Fujioka, A. and Duman, R. S. (2004). Activation of cAMP signaling facilitates the morphological maturation of newborn neurons in adult hippocampus. *Neuroscience* **24**, 319-328.

Fukui, M., Nakamichi, N., Yoneyama, M., Ozawa, S., Fujimori, S., Takahata, Y., Nakamura, N., Taniura, H. and Yoneda, Y. (2008). Modulation of cellular proliferation and differentiation through GABA(B) receptors expressed by undifferentiated neural progenitor cells isolated from fetal mouse brain. *J. Cell. Physiol.* **216**, 507-519.

Gassmann, M., Shaban, H., Vigot, R., Sansig, G., Haller, C., Barbieri, S., Humeau, Y., Schuler, V., Müller, M., Kinzel, B. et al. (2004). Redistribution of GABAB(1) protein and atypical GABAB responses in GABAB(2)-deficient mice. *J. Neurosci.* **24**, 6086-6097.

Ge, S., Goh, E. L., Sailor, K. A., Kitabatake, Y., Ming, G. L. and Song, H. (2006). GABA regulates synaptic integration of newly generated neurons in the adult brain. *Nature* **439**, 589-593.

Haller, C., Casanova, E., Müller, M., Vacher, C. M., Vigot, R., Doll, T., Barbieri, S., Gassmann, M. and Bettler, B. (2004). Floxed allele for conditional inactivation of the GABAB(1) gene. *Genesis* **40**, 125-130.

Hattiangady, B. and Shetty, A. K. (2008). Aging does not alter the number or phenotype of putative stem/progenitor cells in the neurogenic region of the hippocampus. *Neurobiol. Aging* **29**, 129-147.

Haydar, T. F., Wang, F., Schwartz, M. L. and Rakic, P. (2000). Differential modulation of proliferation in the neocortical ventricular and subventricular zones. *J. Neurosci.* **20**, 5764-5774.

Hirrlinger, P. G., Scheller, A., Braun, C., Hirrlinger, J. and Kirchhoff, F. (2006). Temporal control of gene recombination in astrocytes by transgenic expression of the tamoxifen-inducible DNA recombinase variant CreERT2. *Glia* **54**, 11-20.

Jagasia, R., Steib, K., Englberger, E., Herold, S., Faus-Kessler, T., Saxe, M., Gage, F. H., Song, H. and Lie, D. C. (2009). GABA-cAMP response element-binding protein signaling regulates maturation and survival of newly generated neurons in the adult hippocampus. *J. Neurosci.* **29**, 7966-7977.

Jessberger, S. and Gage, F. H. (2008). Stem-cell-associated structural and functional plasticity in the aging hippocampus. *Psychol. Aging* **23**, 684-691.

Jhaveri, D. J., Mackay, E. W., Hamlin, A. S., Marathe, S. V., Nandam, L. S., Vaidya, V. A. and Bartlett, P. F. (2010). Norepinephrine directly activates adult hippocampal precursors via beta3-adrenergic receptors. *J. Neurosci.* **30**, 2795-2806.

Kaupmann, K., Huggel, K., Heid, J., Flor, P. J., Bischoff, S., Mickel, S. J., McMaster, G., Angst, C., Bittiger, H., Froestl, W. et al. (1997). Expression cloning of GABA(B) receptors uncovers similarity to metabotropic glutamate receptors. *Nature* **386**, 239-246.

Kempermann, G., Jessberger, S., Steiner, B. and Kronenberg, G. (2004). Milestones of neuronal development in the adult hippocampus. *Trends Neurosci.* **27**, 447-452.

Kronenberg, G., Reuter, K., Steiner, B., Brandt, M. D., Jessberger, S., Yamaguchi, M. and Kempermann, G. (2003). Subpopulations of proliferating cells of the adult hippocampus respond differently to physiologic neurogenic stimuli. *J. Comp. Neurol.* **467**, 455-463.

Kuner, R., Köhr, G., Grünewald, S., Eisenhardt, G., Bach, A. and Kornau, H. C. (1999). Role of heteromer formation in GABAB receptor function. *Science* **283**, 74-77.

Liu, X., Wang, Q., Haydar, T. F. and Bordey, A. (2005). Nonsynaptic GABA signaling in postnatal subventricular zone controls proliferation of GFAP-expressing progenitors. *Nat. Neurosci.* **8**, 1179-1187.

Lugert, S., Basak, O., Knuckles, P., Haussler, U., Fabel, K., Götz, M., Haas, C. A., Kempermann, G., Taylor, V. and Giachino, C. (2010). Quiescent and active hippocampal neural stem cells with distinct morphologies respond selectively to physiological and pathological stimuli and aging. *Cell Stem Cell* **6**, 445-456.

Lugert, S., Vogt, M., Tchorz, J. S., Müller, M., Giachino, C. and Taylor, V. (2012). Homeostatic neurogenesis in the adult hippocampus does not involve amplification of Ascl1(high) intermediate progenitors. *Nat. Commun.* **3**, 670.

Malitschek, B., Rüegg, D., Heid, J., Kaupmann, K., Bittiger, H., Fröstl, W., Bettler, B. and Kuhn, K. (1998). Developmental changes of agonist affinity at GABABR1 receptor variants in rat brain. *Mol. Cell. Neurosci.* **12**, 56-64.

Martin, S. C., Russek, S. J. and Farb, D. H. (1999). Molecular identification of the human GABABR2: cell surface expression and coupling to adenylyl cyclase in the absence of GABABR1. *Mol. Cell. Neurosci.* **13**, 180-191.

Masiulis, I., Yun, S. and Eisch, A. J. (2011). The interesting interplay between interneurons and adult hippocampal neurogenesis. *Mol. Neurobiol.* **44**, 287-302.

Palmer, T. D., Takahashi, J. and Gage, F. H. (1997). The adult rat hippocampus contains primordial neural stem cells. *Mol. Cell. Neurosci.* **8**, 389-404.

Parent, J. M. (2007). Adult neurogenesis in the intact and epileptic dentate gyrus. *Prog. Brain Res.* **163**, 529-540, 817.

Pérez-Garci, E., Gassmann, M., Bettler, B. and Larkum, M. E. (2006). The GABAB1b isoform mediates long-lasting inhibition of dendritic Ca2+ spikes in layer 5 somatosensory pyramidal neurons. *Neuron* **50**, 603-616.

Petrik, D., Lagace, D. C. and Eisch, A. J. (2012). The neurogenesis hypothesis of affective and anxiety disorders: are we mistaking the scaffolding for the building? *Neuropharmacology* **62**, 21-34.

Salazar, P., Velasco-Velázquez, M. A. and Velasco, I. (2008). GABA effects during neuronal differentiation of stem cells. *Neurochem. Res.* **33**, 1546-1557.

Schuler, V., Lüscher, C., Blanchet, C., Klix, N., Sansig, G., Klebs, K., Schmutz, M., Heid, J., Gentry, C., Urban, L. et al. (2001). Epilepsy, hyperalgesia, impaired memory, and loss of pre- and postsynaptic GABA(B) responses in mice lacking GABA(B(1)). *Neuron* **31**, 47-58.

Song, J., Zhong, C., Bonaguidi, M. A., Sun, G. J., Hsu, D., Gu, Y., Meletis, K., Huang, Z. J., Ge, S., Enikolopov, G. et al. (2012). Neuronal circuitry mechanism regulating adult quiescent neural stem-cell fate decision. *Nature* **489**, 150-154.

Tchorz, J. S., Suply, T., Ksiazek, I., Giachino, C., Cloëtta, D., Danzer, C. P., Doll, T., Isken, A., Lemaistre, M., Taylor, V. et al. (2012). A modified RMCE-compatible Rosa26 locus for the expression of transgenes from exogenous promoters. *PLoS ONE* **7**, e30011.

Tozuka, Y., Fukuda, S., Namba, T., Seki, T. and Hisatsune, T. (2005). GABAergic excitation promotes neuronal differentiation in adult hippocampal progenitor cells. *Neuron* **47**, 803-815.

Ulrich, D. and Bettler, B. (2007). GABA(B) receptors: synaptic functions and mechanisms of diversity. *Curr. Opin. Neurobiol.* **17**, 298-303.

Ursino, M. G., Vasina, V., Raschi, E., Crema, F. and De Ponti, F. (2009). The beta3-adrenoceptor as a therapeutic target: current perspectives. *Pharmacol. Res.* **59**, 221-234.

Vigot, R., Barbieri, S., Bräuner-Osborne, H., Turecek, R., Shigemoto, R., Zhang, Y. P., Luján, R., Jacobson, L. H., Biermann, B., Fritschy, J. M. et al. (2006). Differential compartmentalization and distinct functions of GABAB receptor variants. *Neuron* **50**, 589-601.

Wang, D. D. and Kriegstein, A. R. (2009). Defining the role of GABA in cortical development. *J. Physiol.* **587**, 1873-1879.

Wang, L. P., Kempermann, G. and Kettenmann, H. (2005). A subpopulation of precursor cells in the mouse dentate gyrus receives synaptic GABAergic input. *Mol. Cell. Neurosci.* **29**, 181-189.

Development