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RESEARCH ARTICLE

STEM CELLS AND REGENERATION

# GABA suppresses neurogenesis in the adult hippocampus through $GABA_B$ receptors

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## 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; 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 $GABA_A$ 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.

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$GABA_B$ 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-Garci 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 (Fukui 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 $GABA_B$-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,

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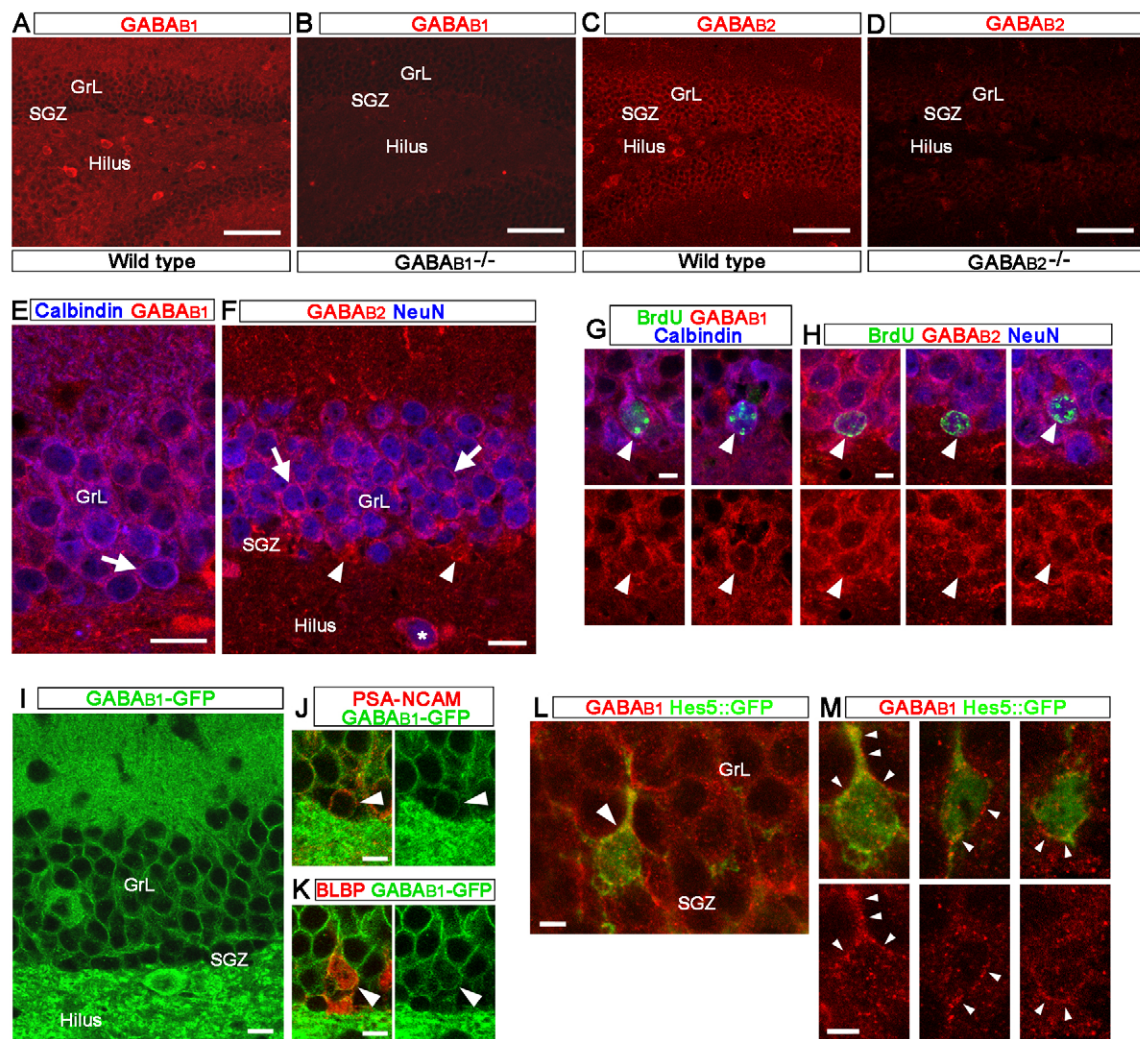


**Fig. 1. $GABA_B$ receptors are expressed and active in the mouse adult hippocampal neurogenic niche.** (A-D) Immunostaining for $GABA_{B1}$ (A,B) and $GABA_{B2}$ (C,D) receptor subunits expression in the adult hippocampal DG of wild-type (A,C) and $GABA_{B1}^{-/-}$ (B) or $GABA_{B2}^{-/-}$ (D) mice. $GABA_{B1}$ 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

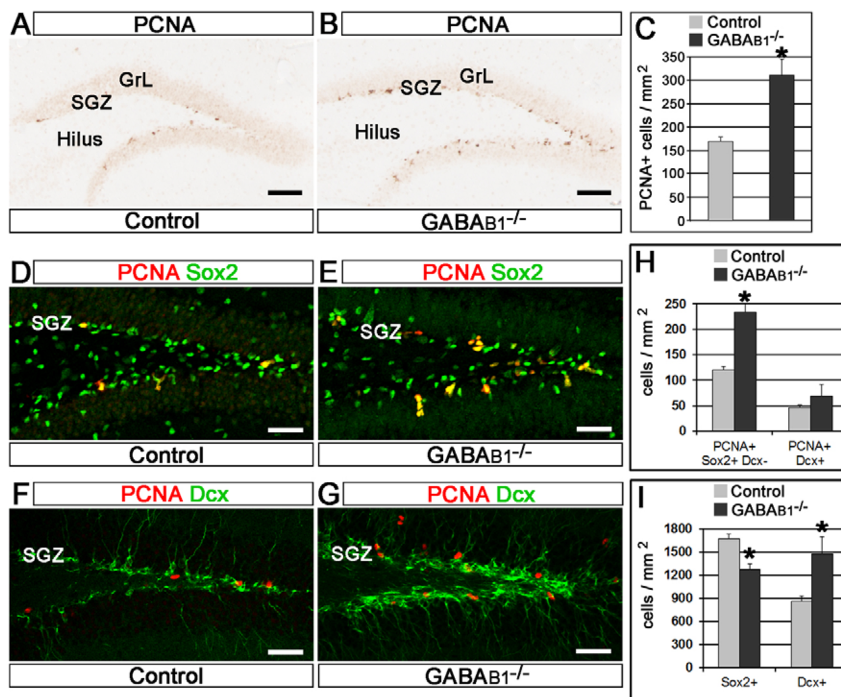


**Fig. 2. Increased proliferation in the hippocampus of $GABA_{B1}$-deficient mice.** (A,B) Representative images of proliferating $PCNA^+$ cells in the adult hippocampal DG of $GABA_{B1}^{-/-}$ and wild-type control mice. (C) The density of $PCNA^+$ proliferating cells is increased in mutant mice (control 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).

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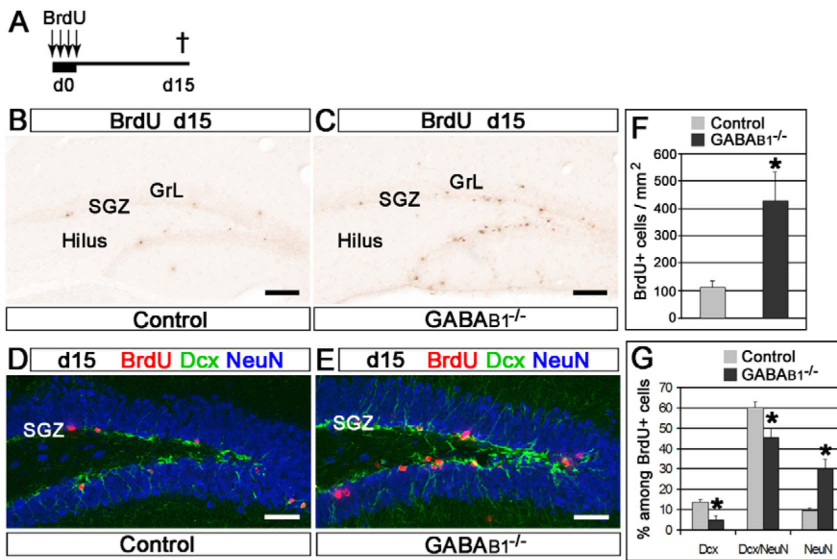


**Fig. 3. Increased differentiation in the hippocampus of $GABA_{B1}$-deficient mice.** (A) BrdU was injected intraperitoneally four times on day 0 (d0) to label newly generated cells and the mice were sacrificed (†) 15 days later (d15). (B,C) Representative images of $BrdU^+$ cells in the hippocampus 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

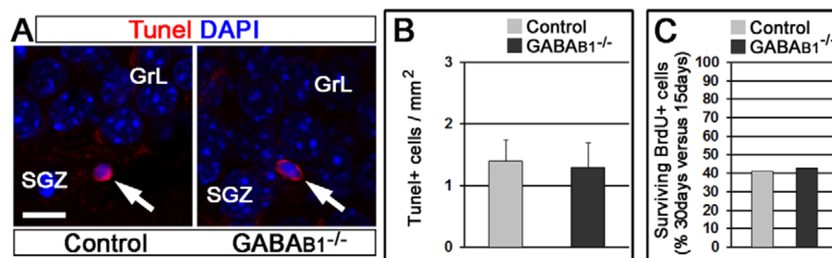


**Fig. 4. Apoptosis and cell survival are not affected in the SGZ of $GABA_{B1}$-deficient mice.** (A) Representative images showing pyknotic $Tunel^+$ cells in the SGZ and GrL of $GABA_{B1}^{-/-}$ and wild-type control mice. (B) The density of $Tunel^+$ cells in the adult hippocampus of $GABA_{B1}^{-/-}$ 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.



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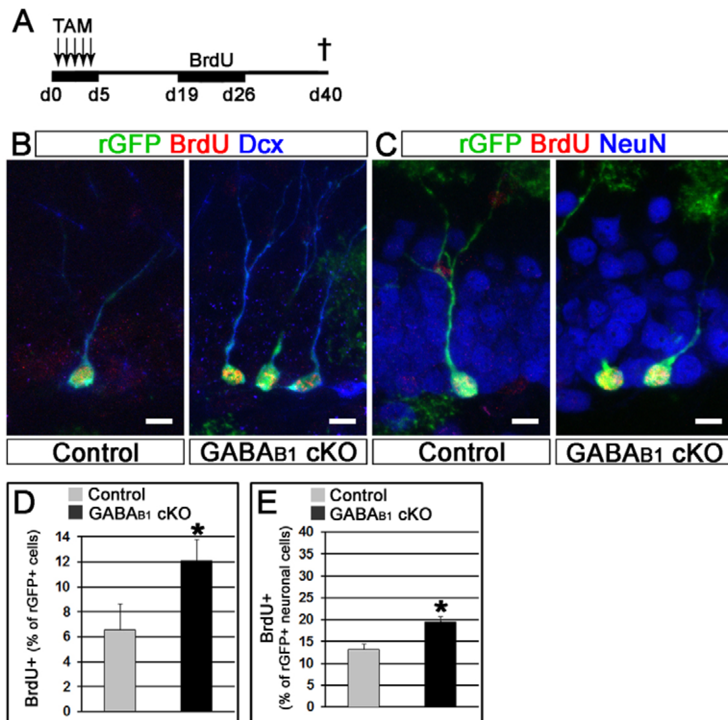


**Fig. 5. $GABA_{B1}$ deficiency cell-autonomously affects adult neurogenesis.** (A) Tamoxifen (TAM) and BrdU induction regimes in *GFAP::CreER*$^{T2}$, rGFP and $GABA_{B1}$$^{lox511/lox511}$ conditional knockout (cKO) mice or $GABA_{B1}$$^{lox511/+}$ control mice. TAM was injected once per day for five 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

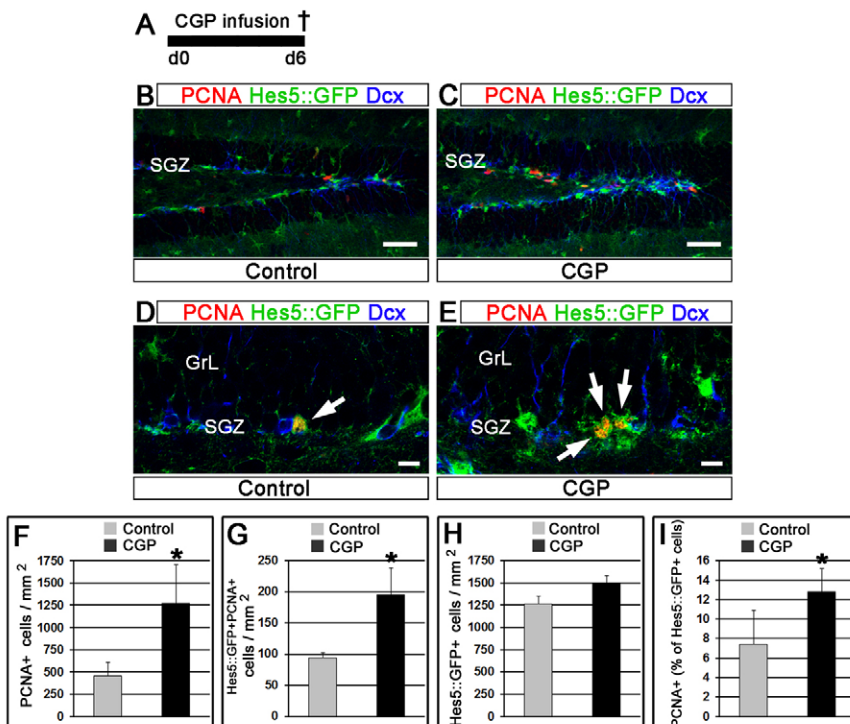


**Fig. 6. Infusion of $GABA_B$ antagonist activates adult hippocampal NSCs.** (A) $GABA_B$ antagonist induction regime. CGP54626A (CGP) was infused for 6 days into the hippocampus of adult *Hes5::GFP*$^+$ mice. The mice were sacrificed (†) at day 6 (d6). (B,C) Representative images of proliferating cells ($PCNA^+$), 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.

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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% $H_2O_2$ 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.).

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#### 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.

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#### Supplementary material

Supplementary material available online at
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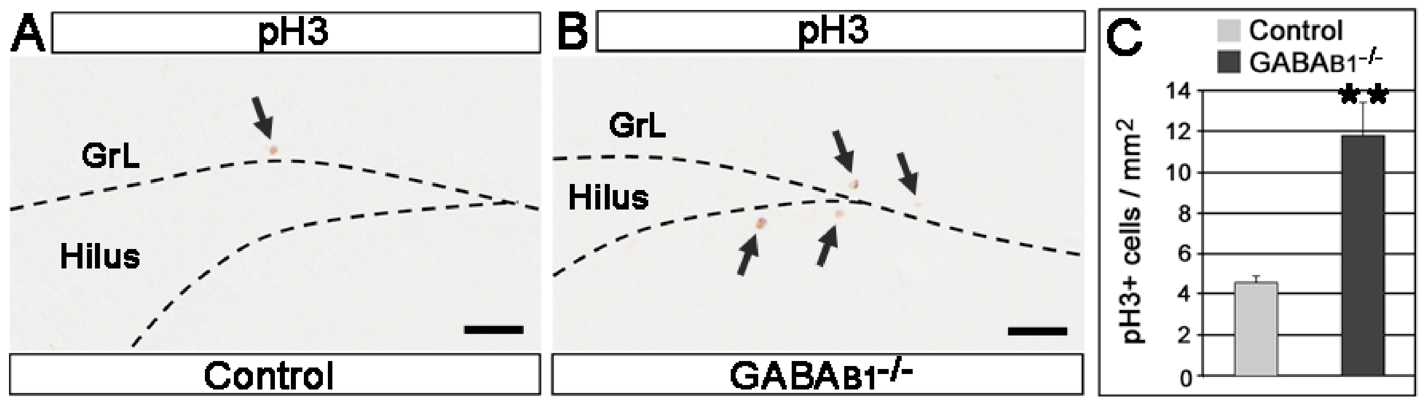


**Fig. S1. Increased proliferation in the hippocampus of $GABA_{B1}$ deficient mice.** (A, B) Representative images of mitotic $pH3^+$ cells in $GABA_{B1}^{-/-}$ and wild-type control mice. (C) The density of $pH3^+$ cells is increased in mutant mice (control 4.7±0.22; $GABA_{B1}^{-/-}$ 11.8±1.8; n=8). GrL, granule cell layer. t-test: **$p<0.01$. Error bars indicate SEM. Scale bars, 50 μm

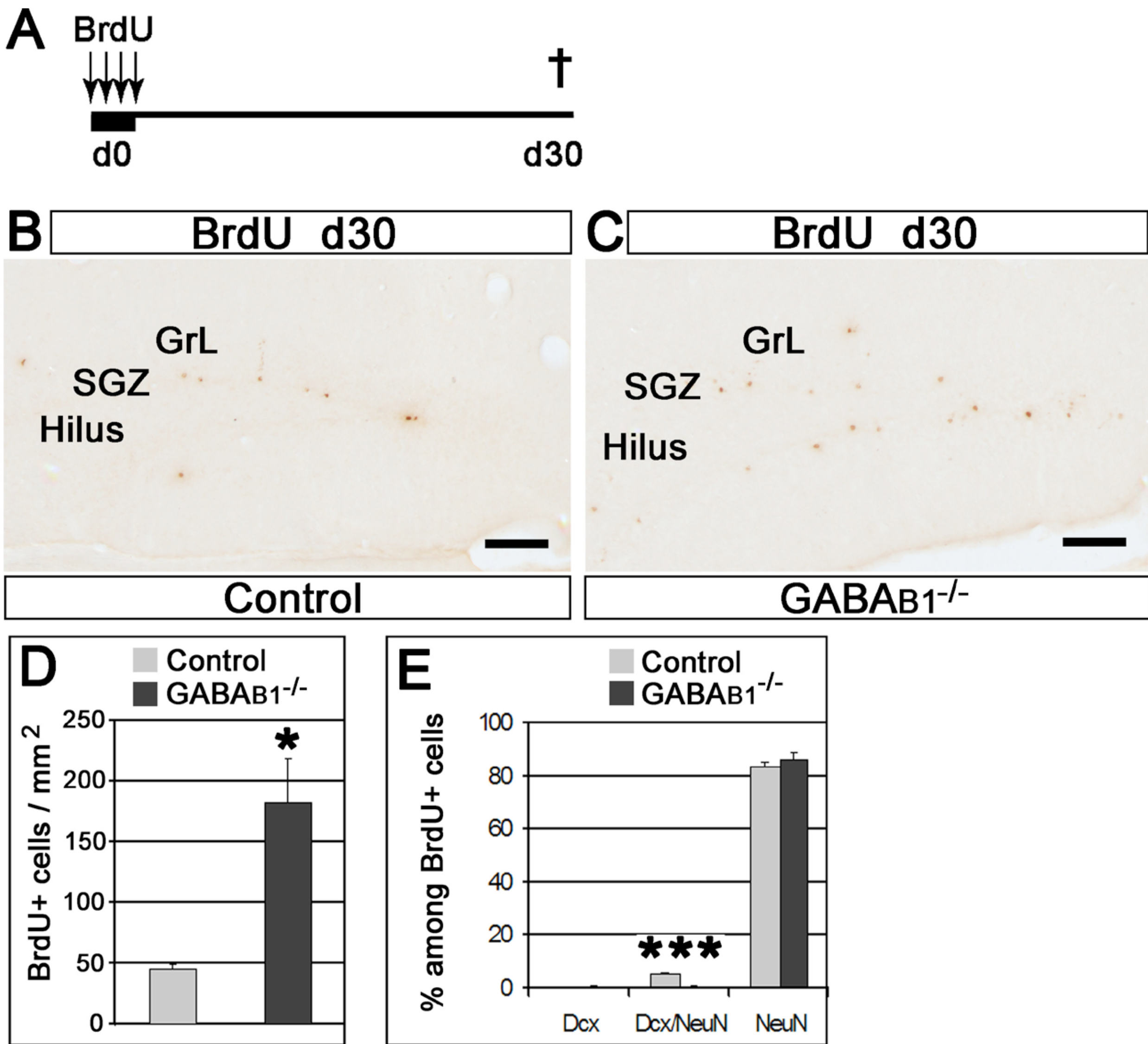


**Fig. S2. Increased neurogenesis in the hippocampus of $GABA_{B1}$ deficient mice.** (A) BrdU was injected intraperitoneally four times a day 0 (d0) to label newly generated cells and the mice were killed (†) 30 days later (d30). (B, C) Representative images of $BrdU^+$ newly generated cells in the adult hippocampus of $GABA_{B1}^{-/-}$ and control mice at day 30. (D) The density of long-term surviving $BrdU^+$ newborn cells is increased 4-fold in $GABA_{B1}$ mutant mice (control 46±3.2; $GABA_{B1}^{-/-}$ 183±36; n=5). (E) Thirty days after BrdU injection, most labeled-newborn cells terminally differentiated into $NeuN^+$ neurons. A few $BrdU^+$ cells still expressed Dcx in control, but not in $GABA_{B1}$ mutant mice (control 5.2±0.49; $GABA_{B1}^{-/-}$ 0.55±0.6; n=5). SGZ, subgranular zone; GrL, granule cell layer. t-test: *p<0.05, ***p<0.001. Error bars indicate SEM. Scale bars, 100 μm.

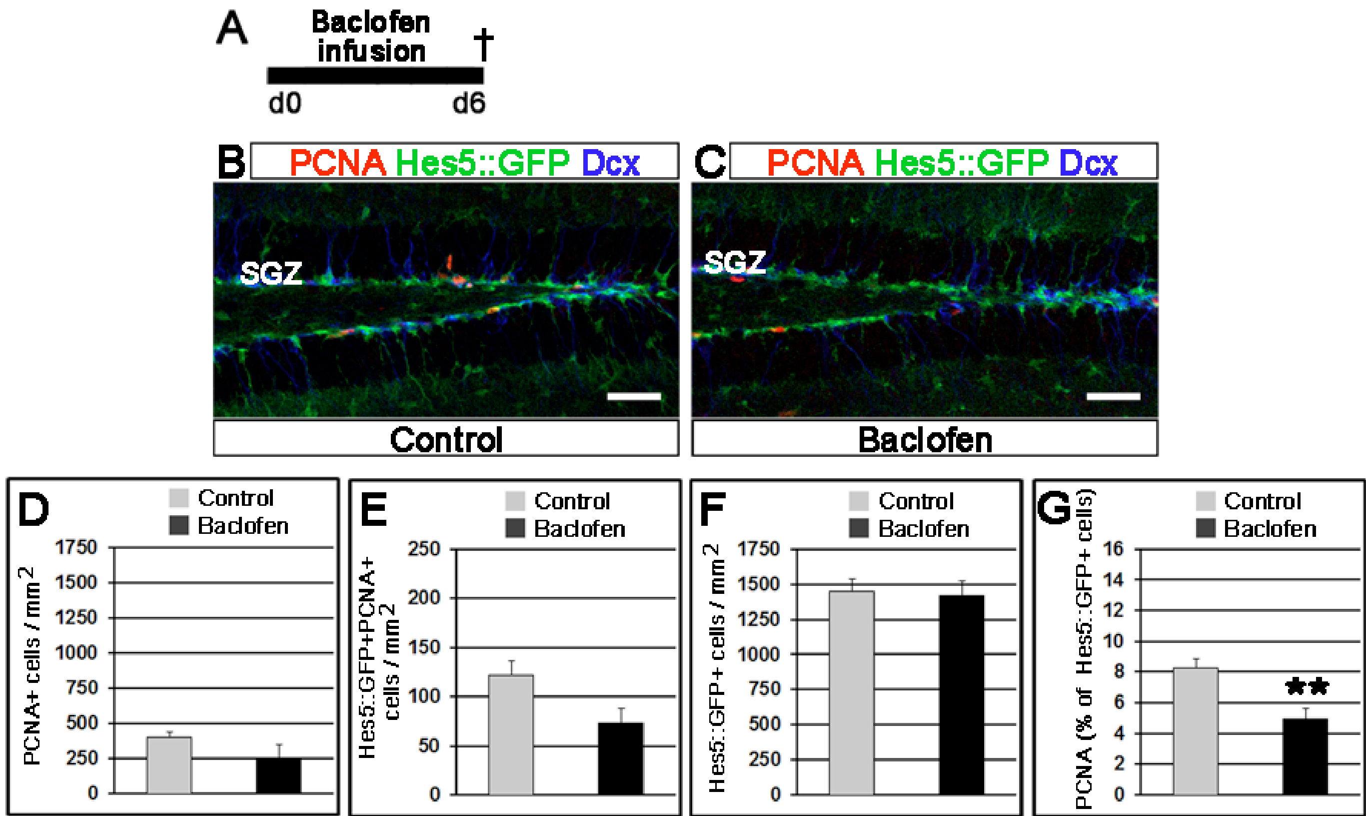


**Fig. S3. Infusion of $GABA_B$ agonist inhibits adult hippocampal NSC proliferation.** (A) $GABA_B$ agonist induction regime. Baclofen was infused for six days into the hippocampus of adult *Hes5::GFP*$^+$ mice. The mice were killed (†) at day 6 (d6). (B, C) Representative images of proliferating cells (PCNA$^+$), neuroblasts (Dcx$^+$) and NSCs (*Hes5::GFP*$^+$) in the SGZ of Baclofen and control (saline) infused mice. (D) The density of PCNA$^+$ proliferating cells is not significantly changed in Baclofen-treated mice (control 398±37; CGP 255±94; n=5). (E) The density of proliferating (PCNA$^+$) *Hes5::GFP*$^+$ cells is slightly but not significantly decreased in Baclofen treated mice (control 121±15; Baclofen 73±16; n=5; p=0.057). (F) The *Hes5::GFP*$^+$ population does not change in size after Baclofen treatment (control 1451±87; Baclofen 1418±105; n=5). (G) The proportion of *Hes5::GFP*$^+$ cells that proliferate (PCNA$^+$) decreases after Baclofen treatment (control 8.2±0.6; Baclofen 4.9±0.7; n=5). SGZ, subgranular zone. t-test: **p<0.01. Error bars indicate SEM. Scale bars, 50 µm.