Cell cycle restriction by histone H2AX limits proliferation of adult neural stem cells

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Edited by Pasko Rakic, Yale University, New Haven, CT, and approved February 25, 2011 (received for review October 6, 2010)

Adult neural stem cell proliferation is dynamic and has the potential for massive self-renewal yet undergoes limited cell division in vivo. Here, we report an epigenetic mechanism regulating proliferation and self-renewal. The recruitment of the PI3K-related kinase signaling pathway and histone H2AX phosphorylation following GABA<sub>A</sub> receptor activation limits subventricular zone proliferation. As a result, NSC self-renewal and niche size is dynamic and can be directly modulated in both directions pharmacologically or by genetically targeting H2AX activation. Surprisingly, changes in proliferation have long-lasting consequences on stem cell numbers, niche size, and neuronal output. These results establish a mechanism that continuously limits proliferation and demonstrates its impact on adult neurogenesis. Such homeostatic suppression of NSC proliferation may contribute to the limited self-repair capacity of the damaged brain.

DNA damage response | subventricular zone astrocytes | self-repair

Neural stem cells (NSCs) reside in discrete germinal regions of the adult brain and self-renew to generate neurons throughout life. Within the largest neurogenic region, the subventricular zone (SVZ) of the lateral ventricles, NSCs (also referred to as type B cells) are localized to a germinal zone that also contains progeny cells with more restricted potential. Type B cells display ultrastructural and molecular characteristics of astroglial cells, including the expression of GFAP and GLAST (1, 2). The lineage from NSCs to neurons includes the immediate progeny, referred to as transit amplifying cells, which in turn generate young neurons, neuroblasts, that migrate to the olfactory bulb (OB), where they integrate as local interneurons (3). Following damage to the brain, such as stroke, SVZ NSCs have been shown to migrate to the site of injury, to differentiate into neurons and functionally integrate into the brain parenchyma (4, 5).

Adult neurogenesis is dynamically regulated throughout life and is affected by aging, environmental challenges, exercise, stress, and neuropathological conditions (6, 7). The net extent of neurogenesis is the result of several processes including stem cell proliferation, differentiation, and the survival of newly generated cells. Consistently, the number of neurons migrating to selected brain regions, differentiating into neurons, and integrating into normal neuronal circuitry is correlated to, and possibly limited by, SVZ proliferation (8). Because NSCs are relatively quiescent whereas progenitor cells are actively proliferating, a small increase in stem cell numbers might be sufficient to lead to large differences in neuronal output. Elevated neurogenesis during neuropathological conditions is associated with enhanced stem and progenitor cell self-renewal (4, 8), and furthermore, depletion of the transit amplifying progenitors and neuroblasts in the SVZ leads to a marked increase in stem cell proliferation (9), suggesting that the relatively slow endogenous proliferation of adult NSCs is controlled by a homeostatic mechanism such that, when there are few stem and progenitor cells in the niche, they proliferate faster than if there are many cells. Hence, adult NSCs are endowed with enhanced self-renewal and cellular output under certain circumstances, possibly as a result of disinhibition of stem cell self-renewal.

GABA signaling represents one possible mechanism that regulates adult neurogenesis. Depolarizing neuroblasts in the SVZ stem cell niche nonsynthetically activate GABA<sub>A</sub> receptors (GABA<sub>A</sub>Rs) in GFAP<sup>+</sup> SVZ cells. The application of GABA<sub>R</sub> antagonist to slices containing the SVZ for 18 h leads to an increase in proliferating cells as visualized by BrdU incorporation (10). Surprisingly, GABA has also been shown to induce an epigenetic signal by PI3 kinase-related kinases (PIKKs) ATM- and ATR-dependent phosphorylation of the histone variant H2AX at Ser-139 (γH2AX), resulting in a reduced rate of proliferation in embryonic stem cells (11). Here, we asked whether a ligand-activated epigenetic mark of histone H2AX plays a critical role in stem cell self-renewal, niche size, and neurogenesis in the adult brain.

Results

SVZ NSCs Express Components Required to Respond to GABA and Display GABA<sub>R</sub>-Dependent Proliferation via H2AX in Vitro. We developed a simple strategy using GFAP-GFP transgenic mice to identify SVZ cell types as previously described, based on microdissecting the SVZ and amplifying and analyzing primary neurospheres in vitro, which allowed identification of GFAP<sup>+</sup> relatively quiescent stem cell clones (GFP<sup>high</sup>) versus transit amplifying cell clones (GFP<sup>+</sup>) (12). Consistent with adult NSCs, GFP<sup>high</sup> neurospheres appearing after 4 d in SVZ cultures expressed S100β, Sox2, SSEA1, and nestin (Fig. S1A). mRNA of all necessary components for functional GABA<sub>A</sub>Rs were found in GFP<sup>high</sup> neurospheres, including at least one α, β, and γ subunit, with the β3 subunit being the predominant β subunit present, compared with the broader expression pattern seen in GFP<sup>-</sup> neurospheres (Fig. S1B). mRNAs for the GABA synthesizing enzymes GAD65 and GAD67 were also detected (Fig. S1B), and immunohistochemical staining confirmed the expression of GAD65/67, GABA<sub>A</sub>R β3, and GABA<sub>A</sub>R in these primary GFPhigh neurospheres (Fig. S1C).

We next tested if activation or inhibition of GABA<sub>A</sub>R signaling, by using the receptor antagonist muscimol or antagonist bicuculline respectively, could have direct effects on proliferation of primary GFP<sup>+</sup> NSCs. Dissociated cells from microdissected SVZs were placed in culture and stem cell clones displayed GABA<sub>A</sub>R-dependent incorporation of the BrdU analogue 5-ethynyl-2′-deoxyuridine (EdU) along with opposing, but also GABA<sub>A</sub>R-
However, muscimol (4 d) led to a marked reduction of clone size in acute (1.5 h) treatment. LV, lateral ventricle; CPu, caudate putamen.

Induction of H2AX phosphorylation in NSCs and the SVZ following GABAAR signaling activation. (A) γH2AX immunostaining and EdU incorporation (30 min) in control (Ctr), muscimol (Mus), and bicuculline (Bic)-treated clones (2.5 h). (B) EdU incorporation (30 min) in clones grown from H2AX WT and H2AX-null mutant (KO) mice following single acute (2.5 h) treatment (n = 4). (C) γH2AX immunohistochemistry in the SVZ following acute (1.5 h) i.p. muscimol or bicuculline treatment. (D) γH2AX immunohistochemistry in cells positive for GFAP (arrows) or Tuj1 (arrowheads) in SVZ following acute (1.5 h) treatment. LV, lateral ventricle; CPu, caudate putamen striatum. (Scale bars: A, 20 μm; C, 50 μm; D, 10 μm.)

dependent, regulation of H2AX phosphorylation (Fig. 1A). The number of dividing cells was markedly affected in SVZ clones by GABAAR signaling following acute (2.5 h) treatment, such that muscimol decreased and bicuculline increased numbers of dividing cells (Fig. 1B). This effect was dependent on H2AX, as it was completely abolished in H2AX-deficient cells established from H2AX-null mutant mice (Fig. 1B). Each SVZ generated an average of 241 ± 49 (SD) stem cell clones, and GABAAR activation did not have any effect on maintenance of multipotency or differentiation, as a similar number of clones appeared when SVZ cells were grown in the presence of muscimol (247 ± 57; Fig. S1D). However, muscimol (4 d) led to a marked reduction of clone size (Fig. S1E), suggesting a sustained suppression of proliferation.

Taken together, these in vitro studies demonstrate that NSC numbers can be modulated in both directions by GABAAR signaling in a manner dependent on the presence of histone H2AX.

H2AX Is Necessary for GABAAR-Dependent Modulation of Proliferation of NSCs and Progenitors Within the SVZ Niche. To investigate the role of GABAAR signaling via histone H2AX modifications in vivo, muscimol and bicuculline were administered acutely (1.5 h) to adult mice followed by immunohistochemistry or SVZ microdissection for Western blot analysis. Muscimol rapidly increased γH2AX immunohistochemistry in cells adjacent to the lateral wall of the lateral ventricle but was not significantly noted in neurons or glia in other regions of the brain (Fig. 1C). Western blot analysis confirmed that muscimol increased γH2AX levels and ATM/ATR substrate phosphorylation—the kinases that phosphorylate H2AX (13)—an effect specifically seen in the SVZ niche, as no effects were seen in striatum/caudate putamen (Fig. S2). Muscimol-induced H2AX phosphorylation (γH2AX) in the SVZ was localized to some GFAP+ SVZ cells as well as some tuj1+ neuroblasts (Fig. 1D). Thus, activation of GABAAR induces kinases of the DNA damage response pathway as well as phosphorylation of H2AX specifically in cells of the SVZ niche, demonstrating the potential for a unique epigenetic, H2AX-dependent mechanism of proliferation regulation in this cell population.

If indeed H2AX represents an endogenous mechanism of tonic inhibition of stem cell proliferation, then in H2AX-deficient mice, there should be an overall increase of Ki67+ mitotically active cells (14) and an increase of BrdU incorporating cells in the SVZ. In accordance with this expectation, an increase in Ki67+ and BrdU+ cells per SVZ was observed in H2AX-deficient mice (Fig. 2A and B). We then examined if the effects of this epigenetic mechanism can be acutely enhanced or reduced in vivo by examining the putative role of GABAAR signaling via H2AX modification on proliferation in the SVZ by using BrdU (2 h) incorporation, in conjunction with acute (2.5 h) administration of GABAAR antagonist and agonist. BrdU incorporation in the SVZ was significantly decreased by muscimol and increased by bicuculline (Fig. 2C and D). The requirement of H2AX for GABAAR regulation of SVZ proliferation was confirmed in H2AX-deficient mice, as the effect of agonist and antagonist was abolished in mice lacking H2AX (Fig. 2E and F). We also addressed which cell types in the niche respond to GABAAR signaling. Examination of in vivo effects of muscimol and bicuculline on adult GFAP+ SVZ stem cells revealed a rapid reduction (approximately twofold) or increase of BrdU+GFAP+ double-labeled cells identified by confocal microscopy stack analysis following muscimol and bicuculline, respectively (Fig. 2G and Fig. S3A). BrdU incorporation overall was significantly affected in the SVZ and comparison of the number of BrdU+GFAP+ double-positive cells to the total number of BrdU+ cells per SVZ revealed that the effects were not limited to GFAP+ stem cells (Fig. S3B), indicating effects also on type C transit amplifying progenitors. Similar results were also achieved by analyzing BrdU+GFAP+ nuclei from GFAP-GFP transgenic mice in which double-positive nuclei (Fig. S3C) were quantified per SVZ following acute (2.5 h) muscimol or bicuculline treatment in conjunction with a BrdU pulse (2 h). The results revealed a decreased or increased number of BrdU+GFAP+ cells in the SVZ, respectively (Fig. 2H and Fig. S3D). Hence, these data are consistent with the in vitro effects on NSCs and suggest a physiological role of H2AX phosphorylation for regulating stem cell proliferation in the adult SVZ niche. Taken together, these findings support the conjecture of an endogenous restriction of proliferation in the SVZ niche mediated by modifications of histone H2AX, which is established by GABAAR signaling in the adult brain.
GFAP-GFP mice following treatment. Arrowheads indicate GFP+BrdU+ nuclei. LV, lateral ventricle. (Scale bars:

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BrdU LI (percentage BrdU+ normalized to Ki67+ per SVZ section) following acute treatments in WT (arrows indicate some BrdU+Ki67+ cells. (Fig. 3E) and the number of GFP+ cells (i.e., total NSC pool) as well as GFP+Ki67+ cells (i.e., proliferating NSCs) in the SVZ were increased 4 wk later (Fig. 3 F and G and Fig. S4E). These data suggest that the increased proliferation of NSCs in the SVZ during continuous treatment leads to an increase in the number of NSCs and niche size.

Increases of SVZ NSCs by Long-Term Interference of GABAAR Signaling Results in Sustained Effects on NSC Numbers and Neuronal Output. A number of studies have indicated that stem cells can be identified within the SVZ as a result of their ability to retain BrdU as a result of their slow rate of cell division, and that the differentiated BrdU labeled progenies migrate out of the SVZ (9, 17–20). To examine long-term changes, we staggered chronic treatment (8 d agonist/antagonist) with an 8-d BrdU pulse with a 4-d overlap in between, and analyzed such label-retaining cells (LRCs) 3 wk later (Fig. 3H). Muscimol led to a marked and persistent reduction of LRCs whereas bicuculline caused a significant increase in LRCs that persisted to 3 wk after the end of the treatment (Fig. 3I). To address whether this increase represented a recruitment of quiescent stem cells, we administered BrdU and treatment sequentially for 8 d each, followed by a 3-wk chase period without any treatment (Fig. S5I). We then examined the ability of GABAAR to lead to long-term changes in the number of prelabeled LRC cells in the niche. LRCs significantly increased 3 wk after termination of bicuculline treatment compared with muscimol treatment (Fig. S5B). The effect of bicuculline was comparable to the effect on LRCs in the staggered protocol (Fig. 3H and I), suggesting that bicuculline primarily increases the rate of division of already cycling stem cells with limited recruitment of quiescent stem cells. This conclusion was confirmed by quantifying mitotically active (i.e., Ki67+) and mitotically inactive (i.e., quiescent, Ki67−) LRCs in the staggered experimental paradigm (Fig. S5C). The proportional changes in these populations were consistent following muscimol or bicuculline treatment despite the total number of NSCs being decreased or increased, respectively (Fig. S5 D and E). Thus, our data suggest that GABAAR signaling increases the rate of self-renewal, resulting in long-term changes of stem cell numbers of the SVZ niche extending well beyond the end of the treatment period.

SVZ progenitors differentiate into neuroblasts, which migrate through the rostral migratory stream to the OB and differentiate into neurons (3). We next wanted to examine if sustained pharmacological modulation in vivo over a period of 4 d still affects proliferation and whether this results in changes in stem cell numbers and niche size (i.e., self-renewal). For this purpose, we administered muscimol or bicuculline at doses lower than those that lead to overt behavioral response during the 4-d period (Fig. 3A). Ki67 labeling following continuous muscimol or bicuculline treatment was significantly decreased or increased in the SVZ, respectively (Fig. 3B and Fig. S4B). This effect was largely dependent on H2AX, as only minor effects were seen in H2AX-deficient mice (Fig. 3B). Bicuculline induced corresponding changes of cells in G2/M marked by phosphorylated histone H3 (pH3) compared with muscimol treatment (Fig. S4B), as well as an increase in the number of migrating neuroblasts as seen by an increase of the number of doublecortin+ (DCX+) cells 4 d after continuous bicuculline compared with control and muscimol treatment (Fig. S4 C and D). An increase in NSC numbers and niche size as a result of increased proliferation throughout the 4-d period was suggested by an increase and decrease in SVZ GFAP immunoreactivity following bicuculline and muscimol administration, respectively (Fig. 3 C and D). To directly examine the total pool of the NSC population, we genetically labeled NSCs by using GLAST-CreERT2;CAG-GFP mice (15, 16). Treatment with bicuculline for 8 d was combined with 5 d tamoxifen administration (Fig. 3E), and the number of GFP+ cells (i.e., total NSC pool) as well as GFP+Ki67+ cells (i.e., proliferating NSCs) in the SVZ were increased 4 wk later (Fig. 3 F and G and Fig. S4E). These data suggest that the increased proliferation of NSCs in the SVZ during continuous treatment leads to an increase in the number of NSCs and niche size.

Long-Term Modulation of GABAAR Signaling Has Consequences for NSC Numbers and Niche Size. A number of studies have indicated that stem cells can be identified within the SVZ as a result of their ability to retain BrdU as a result of their slow rate of cell division, and that the differentiated BrdU labeled progenies migrate out of the SVZ (9, 17–20). To examine long-term changes, we staggered chronic treatment (8 d agonist/antagonist) with an 8-d BrdU pulse with a 4-d overlap in between, and analyzed such label-retaining cells (LRCs) 3 wk later (Fig. 3H). Muscimol led to a marked and persistent reduction of LRCs whereas bicuculline caused a significant increase in LRCs that persisted to 3 wk after the end of the treatment (Fig. 3I). To address whether this increase represented a recruitment of quiescent stem cells, we administered BrdU and treatment sequentially for 8 d each, followed by a 3-wk chase period without any treatment (Fig. S5I). We then examined the ability of GABAAR to lead to long-term changes in the number of prelabeled LRC cells in the niche. LRCs significantly increased 3 wk after termination of bicuculline treatment compared with muscimol treatment (Fig. S5B). The effect of bicuculline was comparable to the effect on LRCs in the staggered protocol (Fig. 3H and I), suggesting that bicuculline primarily increases the rate of division of already cycling stem cells with limited recruitment of quiescent stem cells. This conclusion was confirmed by quantifying mitotically active (i.e., Ki67+) and mitotically inactive (i.e., quiescent, Ki67−) LRCs in the staggered experimental paradigm (Fig. S5C). The proportional changes in these populations were consistent following muscimol or bicuculline treatment despite the total number of NSCs being decreased or increased, respectively (Fig. S5 D and E). Thus, our data suggest that GABAAR signaling increases the rate of self-renewal, resulting in long-term changes of stem cell numbers of the SVZ niche extending well beyond the end of the treatment period.

SVZ progenitors differentiate into neuroblasts, which migrate through the rostral migratory stream to the OB and differentiate into neurons (3). We tested whether the persistent changes of stem cell numbers with agonist and antagonist leads to changes in cellular output from the SVZ to the OB. Following 8 d treatment and 3 wk chase (Fig. 3H), the resulting number of BrdU+ cells was analyzed in the granule cell layer (GCL) of the OB. Muscimol decreased, whereas bicuculline increased, neuronal output (Fig. 3 J and K and Fig. S5F), correlating in magnitude with the effects seen on LRCs. Because BrdU also labels transit...
Suppression of NSC Self-Renewal by H2AX in Vivo Is a Limiting Factor for Neurogenesis. Finally, we examined the physiological role of H2AX on NSC numbers and neuronal output. Consistent with the increased proliferative capacity in mice lacking H2AX by BrdU and Ki67 immunohistochemistry (Fig. 2 A and B), we found that administration of BrdU for 8 d followed by a 3-wk chase period without BrdU (Fig. 4D) leads to significantly increased number of LRCs in the absence of H2AX (Fig. 4E). Furthermore, we also found significantly more BrdU-labeled cells in the OB (Fig. 4 F and G and Fig. S8). These results demonstrate a physiological role of H2AX in a mechanism that restricts LRC numbers, which may have direct consequences on neurogenesis and neuronal output.

Discussion
NSCs are maintained by self-renewal throughout life in the adult brain, but the precise molecular mechanisms regulating stem cell pool size have not been fully elucidated. The stem cell niche is believed to be reserved as a specialized local microenvironment that has greater potential for self-renewal than is seen in the unchallenged brain. Neurogenesis in the adult brain actively contributes to odor discrimination and cognitive performance, and the age-related declines of these performances correlate with decreases in neurogenesis (21, 22). It is therefore critical to understand the mechanism controlling NSC numbers and its relation to neurogenesis and integration of new neurons. Our data show that the H2AX signaling pathway represents one mechanism restricting NSC proliferation under physiological conditions. When it has been disinhibited genetically or pharmacologically, larger stem cell numbers and increased niche size with greater cellular output is observed.

Previous work has demonstrated that the multipotent cell identity is maintained by suppression of lineage commitment to neuronal or glial fates, thereby preventing a premature depletion of the stem cell pool size (23–27). Accordingly, several diffusible and membrane-associated factors that regulate stem cell numbers have been identified (28). Most notably, Notch signaling regulates stem cell numbers (29) and can increase proliferation in the SVZ by maintaining expression of the multi-/pluripotency transcription factor Sox2, which regulates expression of Sonic hedgehog, which is required for survival and normal proliferation (29–32). Although activation of Notch modulates cell cycle time (33), it also leads to repression of proneural gene expression and maintenance, specifically of the NSC (34–36). In keeping, enhanced Notch signaling by the vascular niche factor pigment epithelium-derived factor diverts asymmetrical division to production of two self-renewing NSCs in the adult brain (37). An absence of Notch signaling in mice results in a transient increase of proliferation and subsequent depletion caused by premature...
Histone H2AX, activation of H2AX was selectively observed in 15% of the cellular histone H2A is represented by the variant phosphorylation of H2AX in the SVZ. Although between 1% and activation leads to activation of ATM/ATR PIKKs and phospho-immunohistochemistry of OB GCL BrdU+ and NeuN+ neurons of H2AX WT and KO at 29 d. (Fernando et al. PNAS)

A ligand-dependent manner. In these experiments, GABAAR H2AX also participates in determining NSC proliferation in approaches, we show that epigenetic modi...differentiation into neurons (38). By using several different approaches, we show that epigenetic modification of histone H2AX also participates in determining NSC proliferation in a ligand-dependent manner. In these experiments, GABAAR activation leads to activation of ATM/ATR PIKKs and phosphorylation of H2AX in the SVZ. Although between 1% and 15% of the cellular histone H2A is represented by the variant histone H2AX, activation of H2AX was selectively observed in the SVZ NSCs and its immediate progenitors in the SVZ niche.

The effects of GABAAR activation on stem cell proliferation are dependent on H2AX given that the effects were abolished in vitro and in vivo in H2AX-deficient mice. Furthermore, the increased number of proliferating NSCs as well as LRCs in histone H2AX-deficient mice places this signaling pathway as a physiological regulator. This conclusion is consistent with robust short- as well as long-term effects on NSCs by pharmacologically blocking endogenous GABAAR activation. It is notable that the increased neuronal output observed in this study does not seem to be paralleled by a depletion of the stem cells. On the contrary, our data unexpectedly support that it appears as a consequence of increased stem cell niche size, and hence may represent a unique mechanism from previously studied processes regulating NSC proliferation. This conclusion is based on the observed increase of GFAP immunoreactivity, analysis of cell numbers labeled in GLAST-CreERT2;CAG-GFP mice following treatment, and the increased number of proliferating cells in H2AX-null mutant mice. However, identification of new unique markers that would allow independent quantification of the NSC population would be desirable to validate this controversial conclusion.

Our results provide evidence of several unexpected general properties of the adult NSC germinal zone. First, the data on BrdU labeling of NSCs and transit amplifying cells as well as GLAST-CreERT2;CAG-GFP genetic cell tracing labeling only the GFAP+ NSCs population proposes that changes in NSC pool size have direct consequences on neuronal output. Hence, although deposition of new neurons into the brain also depends on survival of newborn progenies and differentiation of neurons, an increase in stem cell numbers is sufficient in itself to increase neuronal output, at least in the normal young undamaged brain. Second, the NSC pool is subjected to a continuous dynamic regulation. We find that this regulation involves a restriction in stem cell niche size by epigenetic mechanisms limiting the rate of proliferation, which has consequences on self-renewal. GABA can be released via phasic release through Ca2+-dependent vesicular exocytosis from neurons and by tonic release, independent of action potentials and vesicles, by permeation through the Bestrophin 1 anion channel in neurons and glia (39). Neuroblasts within the germinal zone release GABA tonically, which acts on GFAP+ progenitors in the SVZ, negatively affecting proliferation (10, 40, 41). Bestrophin 1 gates GABA already at very low (i.e., resting) intracellular Ca2+ concentrations (100 nM) but increases release with increasing Ca2+ concentrations. Therefore, the concentration of ambient GABA in the SVZ could directly reflect cell numbers (i.e., at resting membrane, the tonic release would result in a homeostatic control of SVZ niche size) but would also be greatly affected by altering intracellular Ca2+ concentration in cell populations releasing GABA. Hence, a GABA-dependent homeostatic feedback inhibition controlling self-renewal and stem cell niche size in the adult brain and its disinhibition of the epigenetic marks of histone H2AX may contribute to the observed elevation of NSC proliferation and fast repopulation of immediate progenitors and neuroblasts from NSCs following their depletion (19).

Another unexpected finding is that H2AX-dependent mechanisms also lead to long-term alterations in NSC proliferation and neuronal output. Administration of GABAAR agonist or antagonist over a period of several days resulted in sustained changes for several weeks after the end of treatment, as seen in genetically marked NSCs (i.e., LRCs) in GFAP immunoreactivity and in the number of pHis3+, Ki67+, and DCX+ cells. Therefore, the decreases seen following agonist treatment and increases following antagonist treatment after an 8-d treatment seem not to return to normal levels after 3 wk of recovery. The long-term effect of the H2AX signaling pathway on NSC proliferation and neuronal output could be a result of the slow cell division, and limited differentiation, of NSCs and the resulting delayed adjustment of cell numbers. Nevertheless, the clear increase in NSC numbers several weeks after the end of treatment...
could be a consequence of the nature of the signaling pathway. Activation of H2AX by DNA damage, such as double strand breaks or replication fork stalling, triggers temporary cell cycle arrest, but enforced DNA damage response activation can also lead to a state of irreversible growth arrest, i.e., replicative cellular senescence, at least during transformation induced by oncogene activation (42, 43). The long-lasting effect on NSC's observed in this study is particularly relevant given that the GABAAR is the pharmacological target for many drugs used clinically to treat, for example, anxiety disorders and epilepsy. Furthermore, the new-generation hypnotic drugs including zopiclone are commonly used as treatment for insomnia to promote sleep by enhancing the GABAAR activity (44). Our results highlight important implications of therapeutic agents targeting the GABAAR on self-renewal and neuronal output in the brain, but also identifies a mechanism amenable for pharmacological intervention that can result in increases of NSC numbers and neuronal output.

Materials and Methods

Animals and Tissue Preparation. For primary clonal growth, the SVZ region was microprepared and dissociated as described. Male C57BL/6, GFP-APGF, or H2AX-WT and null (i.e., KO) mice between 8 and 12 wk of age were used. Acute in vivo treatments involved i.p. administration of muscimol (6 mg/kg) or bicuculline (4 mg/kg) (i.e., KO) mice between 8 and 12 wk of age were used. Acute in vivo treatments involved i.p. administration of muscimol (6 mg/kg) or bicuculline (4 mg/kg) whereas chronic treatments involved muscimol and bicuculline (4 mg/kg) as indicated twice daily. Tamoxifen (Sigma) dissolved in corn oil was administered by twice daily 1-mg i.p. injections over 5 d (accumulated dose of 10 mg).

Immunohistochemistry, Quantification, and Statistical Analysis. Immunohistochemistry was performed on 14-μm coronal sections and quantified by counting immunopositive cells from at least six entire lengths of the lateral wall of the lateral ventricle (SVZ) and averaged for each animal replicate. BrdU labeling index (L) was calculated as the percentage of BrdU-normalized to total Ki67* cells per ventricle length (SVZ). Statistical analysis was performed using GraphPad software. Mann-Whitney or unpaired two-tailed tests were consistently used, with an α level of 0.05 for all statistical analysis. *P < 0.05, **P < 0.1, and ***P < 0.001.

ACKNOWLEDGMENTS. We thank Prof. Magdalena Götz for sharing the GLAST-CreERT2/CAG-GFP mice and Helena Samuelson for technical support. This work was supported by Swedish Research Council Linne Grants (Developmental Biology for Regenerative Medicine Grant), Swedish Cancer Foundation, Swedish Child Cancer Foundation, Swedish Brain Foundation, Bertil Hällström Research Foundation, EU FP7 MOLPARK collaborative project, KI Strategic Neuroscience Programme, Wallenberg Scholar Award, ERC Advanced Grant 232675 (to P.E.), and Knut and Alice Wallenberg Foundation (Center for Live Imaging of Cells at Karolinska Institutet). R.F. was supported by the Swedish Research Council. B.E was supported by the Federation of the Societies of Biochemistry and Molecular Biology. M.A. was supported by the Swedish Research Council, Swedish Cancer Foundation, and Swedish Childhood Cancer Foundation.
**Supporting Information**

**Fernando et al. 10.1073/pnas.1014993108**

**SI Materials and Methods**

**Mice, in Vivo Treatments, and Processing.** Male GFAP-GFP (1), H2AX WT and null (KO) (2), and GLASTCreERT2;CAG-GFP (3, 4) mice between 8 and 12 wk of age were consistently used as indicated. All chronic treatments not involving the H2AX-null mutant (KO) mice were conducted in C57BL/6 mice (Scanbur). Acute treatments involved i.p. administration of muscimol (6 mg/kg; Torciris), bicuculline methochloride (4 mg/kg; Torciris), or vehicle (PBS solution) for 1.5 h or 2.5 h in conjunction with BrdU pulse (65 μg/kg) for 2 h as indicated. Chronic treatments involved twice-daily i.p. administration of muscimol or bicuculline (4 mg/kg) for 4 d. For chronic chase experiments, mice were treated as described earlier except for 8 d in conjunction with BrdU drinking water (1 mg/mL) followed by a 3-wk chase period with no treatment. Tamoxifen was dissolved in corn oil and administered to 8-wk GLASTCreERT2;CAG-GFP mice by twice-daily 1-mg i.p. injections over 5 d. At the end time point of all experiments, mice were administered a lethal overdose of isoflurane, followed by transcardial perfusion with 4% paraformaldehyde (PFA) in phosphate buffer, pH 7.5 (30 min). Dissected brains were postfixed in 4% PFA for 6 h, cryoprotected with 10% to 30% sucrose, and frozen rapidly in optimal cutting temperature medium. Tissue was stored at −20 °C until use.

**Clonal Growth and Quantification.** For primary clonal growth, the SVZ region was microdissected and dissociated for 1 h at 37 °C in 0.2 mg/mL papain (Worthington). Clonal growth from single cell suspension was achieved by 4 to 5 d in vitro in DMEM/F12 (Gibco) with B27 supplement without vitamin A (Invitrogen) with 20 ng/mL EGF (recombinant mouse; Nordic Biosite) and bFGF (human; R&D Systems) resuspended every 48 h, at which time GFP^{high} neurospheres were visually distinguished and collected for quantitative RT-PCR or immunocytochemistry. For immunohistochemistry, clones were attached to glass coverslips coated with 5% matrigel (BD Biosciences) during EdU pulse (10 μM, 30 min; Invitrogen), fixed with 4% PFA, and permeabilized in 10% normal donkey serum (NDS) plus 0.3% Tx-100. Following antibody incubations (listed later) EdU incorporation was resolved by using Alexa Fluor azide (Invitrogen) and quantitated relative to DAPI confocal sections through at least six clones for each replicate. For measurements of sphere clone volume, images were threshold-adjusted to represent the size of spheres pictured and then processed with ImageJ for area analysis. More than 100 individual spheres were quantified for each treatment (n = 4). Area values were converted to volume using the formula \( V = \frac{4}{3} \pi \left(\frac{\sqrt[3]{\text{Area}}}{\pi}\right)^{3} \) and normalized to control (i.e., 100). The data were pooled to demonstrate the average volume of spheres in clonal units.

**Western Blot.** SVZ and striatum were isolated from adult mouse brain and lysed in a cold buffer containing NaCl 150 mM, Tris HCl, pH 8, 10 mM, Tx-100 1.5%, sodium orthovanadate 1 mM, β-glycerophosphate 20 mM, and Complete Protease Inhibitor (Roche). The tissue was homogenized in ice with 10 strokes and then sonicated with three cycles of 10 seconds (output 20% and duty cycle 40%) using a Branson Sonifer 450. Lysates were clarified at 10,640 × g for 10 min at 4 °C. The protein concentration of the supernatant was calculated by protein assay (Bio-Rad), and μg of protein extract was used for the Western analysis. We used the following primary antibodies: γH2AX (Abcam), β-actin (Abcam), phospho-(Ser/Thr) ATM-ATR substrate (2851; Cell Signaling). Secondary antibodies were horseradish peroxidase, conjugated anti-rabbit IgG (Sigma), and anti-mouse IgG (GE Healthcare). The signal was detected by using an ECL Plus Western Blotting Detection System (Amersham). Blots were subjected to minimal level adjustments in Photoshop (Adobe) to ensure clarity of comparability of blots from individual experiments.

**Immunohistochemistry, Antibodies, and Quantification.** Slide-mounted coronal sections (14 μm) were washed with PBS solution and nonspecific interactions blocked by 1 h incubation in 5% NDS, 2% BSA, and 0.3% Tx-100 in PBS solution. Antibody incubations were conducted individually and sequentially. Antibodies—rabbit anti-GABA (1:2,000; Sigma), rabbit anti-GABA<sub>R</sub> (1:1,500; Chemicon), rabbit anti-GAD65/67 (1:1,000; Chemicon), goat anti-GFP FITC conjugated (1:200; Abcam), rabbit anti-H2AX (1:500; Abcam), rabbit anti-GFAP (1:1,000; Dako), rabbit anti-ATM/ATR substrate phosphorylation (1:500; Cell Signaling), mouse anti-Tuj1 (1:250; Promega), rabbit anti-S100 (1:500; Dako), rabbit anti-Sox2 (1:500; Abcam), mouse anti-SEEA1 (1:500; Chemicon), mouse anti-nestin (1:500; Abcam), rat anti-BrdU (1:200; Abcam), rabbit anti-Ki67 (1:200; Neomarkers), mouse anti-NeuN (1:1,000; Chemicon), and guinea pig anti-DCX (1:1,000; Chemicon)—were diluted in 0.3% Tx-100, 0.1% BSA, 1% NDS in PBS solution and incubated with sections for 1 to 2 h at room temperature or overnight at 4 °C. Antibodies were visualized with donkey Alexa IgG as appropriate (1:800; Invitrogen) in 2% BSA in PBS solution. Images of SVZ clonal cultures were acquired with an Olympus FluorView-1000 microscope, and all immunohistochemical images were acquired on a confocal Zeiss Axioplan 100M microscope. Labeling index was determined by quantification of the number of BrdU<sup>+</sup> cells counted in 10 representative SVZ sections per replicate, normalized to Ki67<sup>+</sup> cells, and expressed as a percentage, following acute (2.5 h) i.p. administration of muscimol (6 mg/kg) or bicuculline (4 mg/kg) in conjunction with BrdU (65 mg/kg, 2 h) as indicated. Number of BrdU<sup>+</sup>, Ki67<sup>+</sup>, pH3<sup>+</sup>, or DCX<sup>+</sup> cells per SVZ was quantified from single or confocal stacks through the entire lengths of the lateral wall of the six to 10 lateral ventricles (SVZ) per replicate following acute (2.5 h) muscimol (6 mg/kg) or bicuculline (4 mg/kg) or chronic muscimol or bicuculline (4 mg/kg twice daily for 4 d) treatments. For GFAP<sup>+</sup> integrated density analysis following chronic treatment (as described earlier), nonsaturated images from equivalent SVZ regions between treatments were analyzed with ImageJ software to quantify integrated density of GFAP staining per relevant area within each image for each replicate. Threshold adjustments were set to ensure quantification of only positive immunostaining. For persistent changes in stem cell numbers and changes of neuronal output, chronic chase protocols involved BrdU administration (1 mg/mL) in daily drinking water for 8 d in combination with twice-daily injections of muscimol or bicuculline (4 mg/kg) as indicated, followed by a 3-wk chase period without BrdU administration. LRCs in at least 10 entire SVZs were quantified for each replicate. BrdU<sup>+</sup> cells in at least 10 coronal confocal images of the entire GCL region of the OB (defined by NeuN staining) and quantified ImageJ particle and area analysis software. Analysis thresholds were set in order to identify and count only true immunopositive nuclei.

Fig. S1. Activation of functional GABA<sub>A</sub>Rs in SVZ NSC clones decreases sphere size. (A) Representative merged stacks through GFP<sup>high</sup> neurospheres immunostained for S100β, SOX2, SSEA1, and nestin (red) compared with GFP (green) and nuclear DAPI (blue). (B) Relative mRNA expression of GABA<sub>A</sub>R subunits and GABA synthesizing enzymes of GFP<sup>high</sup> and GFP<sup>−</sup> SVZ neurospheres from GFAP-GFP mice determined by quantitative PCR (plus and minus signs indicate relative levels; n = 3, biological triplicates). (C) Immunostaining of GFP<sup>high</sup> clones for GAD65/67, GABA<sub>A</sub>R<sub>β3</sub>, and GABA (red) compared with GFP (green) and nuclear DAPI (blue). (D) Total number of primary GFP<sup>high</sup> clones produced from SVZ cells grown in the presence or absence of muscimol (100 μm twice daily for 4 d; n = 7). (E) Quantification of GFP<sup>high</sup> clone size expressed as volume (n = 7) from D (**P < 0.001). (Scale bars, 20 μm.)
Fig. S2. Muscimol induced activation of PIKK and phosphorylation of H2AX in the SVZ niche. (A) Representative immunohistochemistry of ATM/ATR substrate phosphorylation in GFAP+ cells (arrows) in SVZ segments following acute (1.5 h) treatment. (B) Western blot of PIKK activity and H2AX phosphorylation (γH2AX) in SVZ and striatum following acute (1.5 h) treatment. (C) Quantification of Western blot analyses normalized to β-actin (n = 3). LV, lateral ventricle (**P < 0.01). (Scale bars, 20 μm.)
Fig. S3. Characterization of BrdU incorporating cells in the SVZ following GABA_A signaling activation or interference. (A) Representative confocal stack of GFAP immunohistochemistry in BrdU-pulsed mice. Cells showing close association between BrdU+ nuclei and GFAP immunopositive processes (arrows) were counted as BrdU+GFAP+ cells. (B) Graphical representation showing the numbers of BrdU+GFAP+ positive cells (gray bars) within the total number of BrdU+ cells (full bars) per SVZ following acute treatment. Statistics refers only to BrdU+GFAP+ cells (gray bars) between treatments (n = 4). (C) Representative confocal stack of a BrdU+GFP+ nuclei from BrdU-pulsed GFAP-GFP mice. (D) Quantification of BrdU+GFP+ labeled cells per SVZ following muscimol or bicuculline treatment (2.5 h) and BrdU (2 h) in GFAP-GFP mice (n = 4; *P < 0.05, **P < 0.01, and ***P < 0.001). (Scale bars, 10 μm.)
Fig. S4. Long-term GABA\textsubscript{A}R signaling modulation induces sustained changes in SVZ proliferation. (A) Immunohistochemistry for Ki67 following chronic treatment (4 mg/kg twice daily for 4 d); arrows indicate Ki67\textsuperscript{+} cells. (B) Quantification of pHis3\textsuperscript{+} cells per SVZ following chronic treatment (n = 5). (C) Immunohistochemistry for DCX following chronic treatment. (D) Quantification of DCX\textsuperscript{+} cells per SVZ following chronic treatment (n = 4). (E) Quantification of GFP\textsuperscript{+}Ki67\textsuperscript{+} SVZ cells at 36 d (n = 4) following lineage tracing using GLAST\textsubscript{creERT2};CAG-GFP mice. The experimental protocol is as described in Fig. 3E (*P < 0.05 and **P < 0.01). (Scale bars, 20 \textmu m.)
Fig. S5. Changes in neuronal output following pharmacological or genetic targeting of H2AX. (A) Schematic timeline showing experimental design for B. (B) Quantification of BrdU+ LRCs at 37 d (n = 4). (C) Schematic timeline showing experimental design for D–F, which involved twice-daily muscimol or bicuculline treatment (4 mg/kg) and BrdU administration (1 mg/mL in drinking water) for 8 d. (D) Quantification of total Ki67+BrdU+ double-positive LRC cells per SVZ at 33 d (n = 4). Ki67+BrdU+ LRCs represent the proliferating LRCs at the experimental endpoint. (E) Quantification of total BrdU+ LRCs that were not Ki67+ (Ki67−BrdU+) per SVZ at 33 d (n = 4). Ki67−BrdU+ cells represent the LRCs that are no longer proliferating. (F) Representative low-magnification images of the GCL of the OB (defined by NeuN) comparing BrdU+ cells at 33 d.
Fig. S6. Induction of GFP expression in GLASTcreERT2;CAG-GFP mice. Representative images of the GCL of the OB in GLASTcreERT2;CAG-GFP mice administered corn oil only (no tamoxifen) or mice administered tamoxifen (2 mg/d for 5 d in corn oil) at 1 or 4 wk after administration. In the absence of tamoxifen, no GFP+ cells were observed. The number of GFP+ neurons in the OB progressively increased at 1 and 4 wk, confirming that the GFP+ neurons in the OB are derived from NSC progeny migrating to the bulb, rather than from local induction. (Scale bar, 50 μm.)

Fig. S7. OB GFP+ neurons in GLASTcreERT2;CAG-GFP mice following chronic bicuculline treatment. (A) Quantification of OB GFP+ cells at 36 d (n = 3–4) in GLASTcreERT2;CAG-GFP mice 4 wk following chronic bicuculline (4 mg/kg) or vehicle treatment (Ctr). (B) Representative low-magnification images of the OB GCL at 36 d comparing GFP+ cells from vehicle versus bicuculline treatment. (Scale bars, 50 μm.)
Fig. S8. OBs from WT and H2AX-null mice. Representative low magnification images of the OB GCL comparing BrdU+ nuclei from H2AX WT or KO mice 3 wk following 8 d of BrdU administration (1 mg/mL in drinking water). (Scale bars, 50 μm.)