Neuronal circuitry mechanism regulating adult quiescent neural stem–cell fate decision

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Adult neurogenesis arises from neural stem cells within specialized niches1–3. Neuronal activity and experience, presumably acting on this local niche, regulate multiple stages of adult neurogenesis, from neural progenitor proliferation to new neuron maturation, synaptic integration and survival1–3. It is unknown whether local neuronal circuits can directly impact adult neural stem cells. Here we show that, in the adult mouse hippocampus, nestin-expressing radial glia-like quiescent neural stem cells (RGLs) respond tonically to the neurotransmitter γ-aminobutyric acid (GABA) by means of γ2-subunit-containing GABA\(_A\) receptors. Clonal analysis of individual RGLs revealed a rapid exit from quiescence and enhanced symmetrical self-renewal after conditional deletion of γ2. RGLs are in close proximity to terminals expressing 67-kDa glutamic acid decarboxylase (GAD67) of parvalbumin-expressing (PV\(^+\)) interneurons and respond tonically to GABA released from these neurons. Functionally, optogenetic control of the activity of dentate PV\(^+\) interneurons, but not that of somatostatin-expressing or vasoactive intestinal polypeptide (VIP)-expressing interneurons, can dictate the RGL choice between quiescence and activation. Furthermore, PV\(^+\) interneuron activation restores RGL quiescence after social isolation, an experience that induces RGL activation and symmetrical division4. Our study identifies a niche cell–signal–receptor trio that can control the activation and self-renewal mode of quiescent adult neural stem cells in response to neuronal activity and experience.

Recent genetic lineage-tracing studies have identified nestin-expressing RGLs as quiescent neural stem cells (qNSCs) in the adult mouse hippocampus5–9. In adult nestin–GFP mice6,7, cells expressing green fluorescent protein (GFP\(^+\)) cells in the subgranular zone (SGZ) with radial processes expressed GFAP (glial fibrillary acidic protein) but rarely MCM2 (minichromosome maintenance type 2), indicating quiescence (Supplementary Fig. 1a, b). To assess whether local interneurons regulate adult qNSCs directly by means of neurotransmitter action and experience that induces RGL activation and symmetrical division5, we assessed 5-ethynyl-2′-deoxyuridine (EdU) incorporation and MCM2 expression by RGLs after treatment with diazepam (Supplementary Fig. 2a). We identified RGLs as SGZ cells with nestin\(^+\) radial processes (Fig. 2a). Stereological quantification showed that treatment with diazepam led to a 45% decrease in the number of EdU\(^+\) RGLs compared with vehicle treatment (Fig. 2b). The number of 5′-ethylic acid decarboxylase (GAD67) of parvalbumin-expressing (PV\(^+\)) interneurons and respond tonically to GABA released from these neurons. Functionally, optogenetic control of the activity of dentate PV\(^+\) interneurons, but not that of somatostatin-expressing or vasoactive intestinal polypeptide (VIP)-expressing interneurons, can dictate the RGL choice between quiescence and activation. Furthermore, PV\(^+\) interneuron activation restores RGL quiescence after social isolation, an experience that induces RGL activation and symmetrical division4. Our study identifies a niche cell–signal–receptor trio that can control the activation and self-renewal mode of quiescent adult neural stem cells in response to neuronal activity and experience.

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Figure 1 | Tonic activation of adult quiescent neural stem cells by GABA by means of α\(_S\)β\(_3\)γ\(_2\) GABA\(_A\)Rs. a. Sample traces of whole-cell voltage-clamp recording from GFP\(^+\) RGLs treated with diazepam (1 µM), flumazenil (10 µM), midazolam (10 µM), ETMD (100 nM) or L-655708 (50 µM), followed by BMI (100 µM) to obtain a baseline for normalizing tonic responses for each cell. b. Summary of normalized amplitude of tonic response. Values are means and s.e.m. (n = 4 or 5 cells; all significantly different from the basal condition; P < 0.05; Student’s t-test).

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RGL quiescence at the population level. Percentage of clones with multiple RGLs in cKO mice (Fig. 3e, f) showed decreased percentages of quiescent clones and an increased percentage of RGLs. Treatment with diazepam decreased RGL symmetrical division and genic asymmetrical RGL division in cKO mice (Fig. 3c). Conversely, at 7 days after induction showed increased symmetrical and astroglio-lineage divisions (Fig. 3a, b). Detailed analysis after induction in adult cKO mice compared with controls, indicating RGL symmetry and inhibit symmetrical self-renewal and astrocyte fate choice through γ2-containing GABAARs in maintaining adult NSC quiescence.

Figure 2 | Cell-autonomous role of γ2-containing GABAARs in maintaining adult neural stem cell quiescence. a, b, Diazepam promotes quiescence of nestin+ RGLs in the adult dentate gyrus. a, Sample confocal images of immunostaining of nestin, MCM2, EdU and 4',6-diamidino-2-phenylindole (DAPI). Arrows indicate nestin+ MCM2+ or nestin+ EdU+ RGLs. Scale bars, 50 μm (left) and 10 μm (last column). b, Summaries of stereological quantification of RGL EdU incorporation and MCM2 expression. Values are means and s.e.m. (n = 4 animals; asterisk, P < 0.01; Student’s t-test). c–e, γ2 deletion in individual RGLs leads to their activation. c, Sample confocal images of immunostaining. Scale bars, 10 μm. d, e, Summaries of percentages of RGL clones that were activated (d) and those treated with vehicle or diazepam at 7 days after induction (e) for control (cntl) and cKO mice. Values are means and s.e.m. (n = 4–8 animals; asterisk, P < 0.01; n.s., P > 0.1; Student’s t-test).

Figure 3 | Clonal analysis of RGL fate choice after conditional γ2 deletion in individual RGLs in the adult dentate gyrus. a–d, Short-term effect of γ2 deletion on the activation and fate choice of adult dentate RGLs. a, Sample confocal images of immunostaining for a GFP+ clone indicating symmetrical division at 7 days after induction. Scale bars, 10 μm. b–d, Summaries of percentages of clones indicating symmetrical divisions at 2 and 7 days after induction (b), and percentages of different types of RGL clones (c) and those treated with vehicle or diazepam (d) at 7 days after induction: R + R (two RGLs), R + intermediate progenitor cell (IPC; one RGL and one GFAP+ IPC) and R + A (one RGL and one GFAP+ bushy astrocyte). Values are means and s.e.m. (n = 4–8 animals; asterisk, P < 0.05; n.s., P > 0.1; Student’s t-test).

de, f, Long-term effect (at 30 days after induction) of γ2 deletion on the composition of GFP+ clones in the adult dentate gyrus. e, Sample confocal images of immunostaining for a clone consisting of two GFAP+ cells with radial processes. Scale bars, 10 μm. f, Summary of percentages of different clone types among all GFP+ clones: R, RGL; N, IPC or neuron; A, astrocyte. Values are means and s.e.m. (n = 4–8 animals; asterisk, P < 0.05; two asterisks, P < 0.01; Student’s t-test).
interneurons (Fig. 4a and Supplementary Movie 1). To determine whether PV+ interneurons interact functionally with RGLs, we took an optogenetic approach and used double-floxed (DIO) adeno-associated virus (AAV) to express channelrhodopsin-2 (ChR2) or halorhodopsin (eNpHR3.0) specifically in PV+ interneurons, using adult PV-Cre mice (Supplementary Fig. 4a). Immunostaining and electrophysiology confirmed the specificity and efficacy of AAV-mediated opsins expression in controlling the firing of dentate PV+ interneurons (Supplementary Fig. 4b–e). In acute slices from PV-Cre+/−:nestin–GFP+/−/mice, photoactivation of PV+ interneurons induced synaptic responses in mature dentate granule cells and tonic responses in GFP+ RGLs to GABA (Fig. 4b, c). Furthermore, a decrease in GABA turnover with the GABA transaminase inhibitor vigabatrin (100 μM) drastically increased GFP+ RGL responses to PV+ interneuron activation (Fig. 4c). Together, these results indicate that adult RGLs respond tonically to GABA released from local PV+ interneurons.

To assess the functional impact of PV+ interneuron activity on RGL behaviour, we photoactivated or suppressed PV+ interneurons in the dentate gyrus of adult PV-Cre mice for 5 days (Supplementary Fig. 5a). In comparison with sham treatment without light stimulation, EdU incorporation and MCM2 expression by RGLs were significantly decreased after activation of PV+ interneurons expressing ChR2 tagged with yellow fluorescent protein (ChR2–YFP), resulting in a 53% decrease in RGL activation at the population level (Fig. 4d and Supplementary Fig. 5b). Conversely, suppression of PV+ interneurons expressing eNpHR–YFP led to a 95% increase in RGL activation (Fig. 4d). These results identified PV+ interneurons as a critical niche component and showed that PV+ interneuron activity can dictate the RGL choice between quiescence and activation in the adult dentate gyrus.

Do other subtypes of local interneurons also regulate RGL behaviour in vivo? We developed similar optogenetic strategies to manipulate somatostatin-expressing (SST+) or vasoactive intestinal polypeptide-expressing (VIP+) interneurons (Supplementary Fig. 6a). Both SST+ and VIP+ interneurons showed elaborated processes in the SGZ and hilus region (Supplementary Fig. 6c, d and Supplementary Movie 2), and our procedure labelled greater numbers of SST+ and VIP+ interneurons than PV+ interneurons in the adult dentate gyrus (Supplementary Fig. 6b). Electrophysiological recording of GFP+ RGLs did not detect any tonic or synaptic responses after light-induced activation of SST+ or VIP+ interneurons in acute slices (Supplementary Fig. 6c, d). Functionally, photoactivated or suppressed dentate SST+ or VIP+ interneurons had no effect on EdU incorporation and MCM2 expression by RGLs (Fig. 4e, f and Supplementary Fig. 6e). Thus, coupling of neuronal circuit activity to RGL behaviour seems to be distinctive of PV+ interneurons rather than occurring broadly across different local interneuron subtypes.

Finally, we assessed whether GABA also serves as a niche signal to mediate experience-dependent regulation of RGLs. We subjected mice to a social isolation regime, which decreases neuronal activity in the adult dentate gyrus and was recently shown to promote GABA expansion. Clonal analysis at 7 days after induction showed that, in contrast with group housing, social isolation led to a significant increase in GABA+ RGL activation and symmetrical and astrogenic division, in a similar manner to γ2 signaling. γ2-deficient RGLs showed no additional activation or fate alternation after social isolation (Fig. 5b). At the population level, EdU incorporation and MCM2 expression by RGLs were increased significantly after social isolation (Fig. 5c and Supplementary Fig. 7b, c). PV+ interneuron activation abolished the increase in RGL activation induced by social isolation (Fig. 5b). Thus, dentate PV+ interneurons also mediate experience-dependent regulation of adult qNSts through GABA–γ2 signalling.

Precise control of somatic stem cell activity is essential for the long-term maintenance of tissue homeostasis and needs to be closely linked to tissue demands at any given time. Our study of adult RGLs at both clonal and population levels identified a previously unknown niche mechanism that regulates both adult qNSt activation and self-renewal mode in response to neuronal activity and experience (Supplementary Fig. 8). GABA has been shown to decrease the proliferation of other stem cells and progenitors in vitro, including mouse embryonic stem
PV interneurons are regulated by physiological and pathological conditions, such as ageing, Alzheimer’s diseases, epilepsy, chronic stress, schizophrenia and other severe psychiatric illness, our findings have broad implications.

METHODS SUMMARY

Wild-type (C57BL/6), nestin–GFP<sup>12</sup>, PV-Cre<sup>16</sup>, SST-Cre<sup>16</sup>, VIP-Cre<sup>16</sup>, nestin-CreERT2<sup>17</sup>, z/EGR2<sup>18</sup>, z/Fos<sup>18</sup> were used in the present study. Cre-dependent recombinant AAV<sup>17</sup> was used for interneuron subtype-specific expression of opsins in the adult dentate gyrus. Electrophysiological recordings and analysis were performed as described previously<sup>19</sup>. Immunohistochemistry, confocal imaging and processing were performed as described previously<sup>20</sup>. Stereological quantification was assessed as described previously<sup>21</sup>. All analyses were performed by investigators blind to experimental conditions. All animal procedures were performed in accordance with institutional guidelines.

Full Methods and any associated references are available in the online version of the paper.

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**Supplementary Information** is linked to the online version of the paper at www.nature.com/nature.

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**Author Contributions** J.S. led the project and contributed to all aspects. C.Z., M.A.B., G.J.S., D.H. and K.C. helped with some experiments. Y.G. and S.G. contributed reagents. J.H. provided SST-Cre mice. G.E. provided nestin–GFP mice. K.D. and K.M. provided initial help on optogenetic tools. B.L. provided γ2\(^{\text{f/f}}\) mice. J.S., G.L.M. and H.S. designed experiments and wrote the paper.

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METHODS

Animals, housing, administration of tamoxifen, EDu and AAV, and optogenetic manipulations. The following genetically modified mice and crosses between them were used for electrophysiological analysis: nestin–GFP<sup>fl</sup> (CB57BL/6 background), PV–Cre<sup>T1</sup> (IAX laboratory; stock number 008069; stock name B6;129P2-Pvahl<sup>(IAX)</sup>), SST–Cre<sup>T2</sup> (IAX laboratory; stock number 010344; stock name Ssttm1(cre)Arbr/J, VIP–Cre<sup>T3</sup> (IAX laboratory; stock number 010908; stock name Vip<sup>(mono) Cre</sup>)). The following mice were used for neurogenesis analysis: wild-type (C57BL/6), nestin-CreER<sup>T2</sup>/Z/EG<sup>Cre</sup><sub>fl</sub>/lox<sub>fl</sub><sub>fl</sub> (ref. 27; C57BL/6) and nestin-CreER<sup>T2</sup>/Z/EG<sup>Cre</sup><sub>fl</sub>/lox<sub>fl</sub> (C57BL/6), PV–Cre (B6;129), SST–Cre (B6;129), and VIP–Cre (B6;129). Animals were housed in a standard 14 h light/10 h dark cycle. Socially isolated animals were individually housed immediately after weaning for at least 6 weeks before injection with tamoxifen or EDu, and had free access to food and water. A single dose of tamoxifen (62 mg/kg) was injected intraperitoneally into 6–10-week-old mice as described previously.<sup>9</sup>

For optogenetic manipulations, Cre-dependent recombinant AAV vectors were used based on a DNA cassette carrying two pairs of incompatible loxP sites with the opsin genes (ChR2-H134–mCherry, ChR2-H134–YFP or eNpHR<sup>3.0</sup>–YFP) inserted between lox sites in the reverse orientation as described previously<sup>10</sup> (Supplementary Fig. 4a). The recombinant AAV vectors were serotyped with AAV2/9 for ChR2 (packaged at the University of North Carolina Vector Core) and with AAV9 for eNpHR<sup>3.0</sup> (packaged at the UPenn Vector Core) and with AAV9 for SST–CreERT2 and nestin-CreERT2 (both packaged at University of North Carolina Vector Core). The following final viral concentrations were used for AAV viruses (<i>n</i> = 24–34 (ref. 17; C57BL/6) were pulled between lox sites in the reverse orientation as described previously<sup>17</sup> and inserted into the dengue virus with the following coordinates (in mm): anterioposterior = −2 from bregma; lateral = ±1.5; ventral = 2.2. Fibre optic cannulae (Doric Lenses, Inc.) were implanted at the same injection sites immediately after AAV injection with a dorsal–ventral depth of 1.6 mm from the skull. Animals were then allowed to recover for at least 4 weeks after surgery. For analysis of RGL activation at the population level after optogenetic manipulations, littermates of animals were used and an in vivo light regime was administered 8 h per day for five consecutive days (Supplementary Figs 5a, 6e and 7b). For ChR2–YFP stimulation, flashes of blue light (472 nm; 5 ms at 8 Hz) through the DPSSL laser system (Laser Century Co. Ltd) were delivered <i>in vivo</i> every 5 min for 30 s per trial. For eNpHR–YFP stimulation, continuous yellow light (593 nm) was delivered <i>in vitro</i>. On the fifth day, animals were injected with EDu (41.1 mg per kg body weight) six times with an interval of 2 h. Animals were killed 2 h after the last EDu injection and were processed for immunostaining as described previously.<sup>9</sup>

All animal procedures were performed in accordance with institutional guidelines.

Electrophysiology. Mice were anesthetized and processed for slice preparation as described previously<sup>11</sup>. In brief, brains were quickly removed into the ice-cold solution (in mM: 110 chloride, 2.5 KCl, 1.3 KH<sub>PO</sub><sub>4</sub>, 25.0 NaHCO<sub>3</sub>, 0.5 CaCl<sub>2</sub>, 7 MgSO<sub>4</sub>, 20 dextrose, 1.3 sodium L-ascorbate, 0.6 sodium pyruvate, 10 dextrose, pH 7.4, 320 mOsM), bubbled with 95% O2/5% CO2. Electrophysiological recordings were obtained at 10 kHz. For measuring GABA-induced synaptic currents, 100 μM GABA was applied to the recording chamber and wash solutions contained 30 mCi of 35S-GABA (Perkin Elmer). Data were collected with an Axon 200B amplifier and acquired with a DigiData 1322A (Axon Instruments) at 10 kHz. For measuring GABA-induced responses from GFP<sup>+</sup> RGLs, focal pressure ejection of 200 mM GABA or muscimol through a puffer pipette controlled by a Picospritz (2 s puff at 3–5 lb in −1) was used to activate GABA<sub>Rs</sub> under the whole-cell voltage-clamp. A bipolar electrode (World Precision Instruments) was used to stimulate (0.1 ms duration) the dentate granule cell layer. Low-frequency stimuli (0.1 Hz) and theta bursts (8 Hz with a train of 100 stimuli) were delivered. The stimulus intensity (50 μA) was maintained for all experiments. The following pharmacological agents were used: diazepam (1 μM), NO-711 (10 μM), flumazenil (10 μM), midazolam (10 μM), ETMD (10 μM), L-655708 (50 μM) and vigabatrin (100 μM). All drugs were purchased from Sigma except bicineuline (50 or 100 μM, Tocris).

RGL recordings under optogenetic manipulation in acute brain slices were performed at least 4 weeks after injection with AAV. To stimulate ChR2 in labelled interneurons, light flashes (5 ms at 1, 8 or 100 Hz) were generated by a Lambda DG–4 plus high-speed optical switch with a 300 W Xenon lamp and a 472 nm filter set (Chroma) were delivered to coronal sections through a ×40 objective lens (Carl Zeiss). To stimulate eNpHR in labelled interneurons, continuous yellow light generated by a DG–4 plus system with a 593 nm filter set were delivered to coronal sections across a full high-power (∼40x) field.

Immunohistochemistry, confocal imaging, processing and quantification. For immunostaining with anti-nestin and anti-MCM2, an antigen retrieval protocol was performed by microwaving sections in boiled citric buffer for 7 min as described previously<sup>12</sup>. For γH2AX immunostaining, a weak fixation protocol using live tissues was adopted as described previously<sup>13,14</sup>. For characterization of different interneuron subtypes, the following antibodies were used: anti-PV (Swant; mouse or rabbit; 1:1000 dilution), anti-GAD-67 (Millipore; mouse or rabbit; 1:500 dilution), anti-GAD-65 (Millipore; mouse or rabbit; 1:500 dilution), anti-SST (Millipore; rat; 1:200 dilution) and anti-VIP (Immunostar; rabbit; 1:200 dilution). For clonal analysis, coronal brain sections (40 μm) through the entire dentate gyrus were collected in a serial order, and immunostaining was performed with the following primary antibodies as described previously<sup>15</sup>: anti-GFP (Rockland; goat; 1:500 dilution), anti-nestin (Aves; chick; 1:200 dilution), anti-MCM2 (BD; mouse; 1:500 dilution), anti-GFAP (Millipore; mouse or rabbit; 1:1000 dilution) and anti-P93–NCAM (Millipore; mouse IgM; 1:500 dilution). For quantification of GFP<sup>+</sup> clones at 2 and 7 days after induction, a single GFP<sup>+</sup> RGL was scored as a quiescent clone. Two or more nuclei in a GFP<sup>+</sup> RGL clone were scored as activation. Clonal analysis at 30 days after induction was conducted exactly as described previously<sup>15</sup>. For experiments with diazepam (5 mg kg<sup>−1</sup> body weight; once daily for 5 days), coronal brain sections (40 μm) through the entire dentate gyrus were collected in a serial order. For optogenetic manipulations, sections within a distance of 1.0 mm anterior and 1.0 mm posterior to injection sites were used for quantification, given the estimated light spread in <i> vivo</i>. Immunostaining was performed on every sixth section as described previously<sup>9</sup>. EDu labelling was performed with a Click-iT EDu Alexa Fluor imaging kit (Invitrogen). Images were acquired on a Zeiss LSM 710 confocal system (Carl Zeiss) with a ×40 objective lens (Carl Zeiss). Immunohistochemistry, confocal imaging, processing and quantification. For quantification of EDu<sup>+</sup> or MCM2<sup>+</sup> RGLs, an inverted ‘Y’ shape from anti-nestin staining superimposed on EDu<sup>+</sup> or MCM2<sup>+</sup> nucleus was scored double positive for nestin and EDu or MCM2. All analyses were performed by investigators blind to experimental conditions. Statistical analysis was performed with Student’s <i>t</i>-test.

For generation of movie files, images were serially reconstructed in Reconstruct (J. C. Fiala, NIH), normalized, and deconvolved with Autoquant X2 (Media Cybernetics). Images were then segmented in MATLAB (The Mathworks) using custom code and imported into Imaris (Bitplane). Surface renderings and movies were made using the Surface and Animation functions, respectively, in Imaris (Supplementary Movies 1–3).