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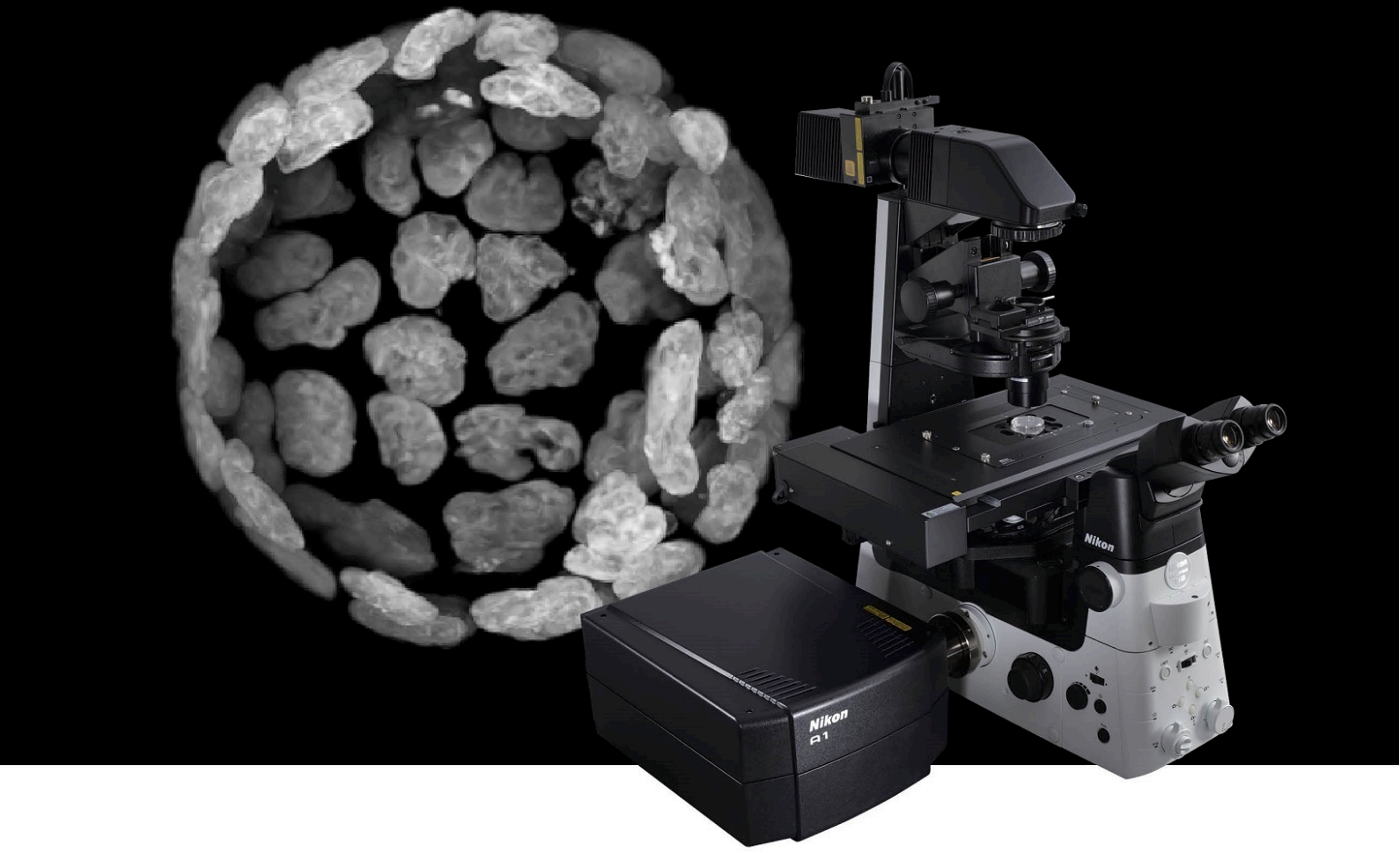

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TISSUE-SPECIFIC STEM CELLS

STEM CELLS

Norepinephrine is a negative regulator of the adult periventricular neural stem cell niche

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Abstract

The limited proliferative capacity of neuroprogenitor cells (NPCs) within the periventricular germinal niches (PGNs) located caudal of the subventricular zone (SVZ) of the lateral ventricles together with their high proliferation capacity after isolation strongly implicates cell-extrinsic humoral factors restricting NPC proliferation in the hypothalamic and midbrain PGNs. We comparatively examined the effects of norepinephrine (NE) as an endogenous candidate regulator of PGN neurogenesis in the SVZ as well as the periventricular hypothalamus and the periaqueductal midbrain. Histological and neurochemical analyses revealed that the pattern of NE innervation of the adult PGNs is inversely associated with their in vivo NPC proliferation capacity with low NE levels coupled to high NPC proliferation in the SVZ but high NE levels coupled to low NPC proliferation in hypothalamic and midbrain PGNs. Intraventricular infusion of NE decreased NPC proliferation and neurogenesis in the SVZ-olfactory bulb system, while pharmacological NE inhibition increased NPC proliferation and early neurogenesis events in the caudal PGNs. Neurotoxic ablation of NE neurons using the Dsp4-fluoxetine protocol confirmed its inhibitory effects on NPC proliferation. Contrarily, NE depletion largely impairs NPC proliferation within the hippocampus in the same animals. Our data indicate that norepinephrine has opposite effects on the two fundamental neurogenic niches of the adult brain with norepinephrine being a negative regulator of adult periventricular neurogenesis. This knowledge might ultimately lead to new therapeutic approaches to influence neurogenesis in hypothalamus-related metabolic diseases or to stimulate endogenous regenerative potential in neurodegenerative processes such as Parkinson's disease.

KEYWORDS
adult neurogenesis, hippocampus, noradrenaline, norepinephrine, olfactory bulb neurogenesis, subventricular zone

1 | INTRODUCTION

The periventricular regions of the entire neuraxis contain neuroprogenitor cells (NPCs) during development, but undergo a gradual loss with age according to a rostro-caudal pattern. Indeed, the subventricular zone of the lateral ventricles (SVZ) remains the main periventricular germinal niche (PGN) in the adult brain producing new neurons of the olfactory bulb.[1] However, numerous studies have demonstrated the existence of NPCs in periventricular regions of the whole ventricular system in the adult brain with a strong rostro-caudal gradual loss of NPC proliferation capacity.[2-4] Recently, the periventricular region bordering the third ventricle (3V) was described as the PGN for adult hypothalamic neurogenesis harboring a heterogeneous population of (sub)ependymal and periventricular adult stem and progenitor cells, including tanycytes as well as subventricular and parenchymal glial cells.[5-10] However, compared to the SVZ of the lateral ventricles, the number of proliferative NPCs within the 3V-PGN and even more caudal in the midbrain periaqueductal (Aq)-PGN is extremely low and local neurogenesis is a rare event.[3,5-7,10] In contrast, functional NPCs can be isolated from the caudal PGNs of the hypothalamic and midbrain region in comparably high numbers with approximately half as much NPC counts in 3V-PGN compared to those of the SVZ as quantified in limiting dilution experiments.[3,11] This discrepancy strongly implies cell-extrinsic factors restricting NPC proliferation in the caudal hypothalamic and midbrain PGNs.

It has been suggested that multiple neurotransmitter systems control adult NPC proliferation within the SVZ linking brain activity to neurogenesis.[12,13] The neurotransmitter norepinephrine (NE)—also called noradrenaline—has thus been implicated in modulating functions of multiple CNS cell types including NPCs of the hippocampal stem cell niche,[14,15] but not yet of the various NPCs of the PGNs. However, its widespread release by non-junctional varicosities from noradrenergic *Locus coeruleus* (LC) neurons projecting to virtually all brain regions except the basal ganglia (striatum) closely adjacent to the SVZ[16,17] make NE a candidate regulator of NPC performance within the caudal PGNs. We therefore dissect the role of NE as a candidate regulator for adult NPC proliferation within the periventricular regions of the adult mouse brain.

Significance statement

Adult neurogenesis is regulated by various factors including growth factors and neurotransmitters such as dopamine. Using a series of experiments designed to stimulate or deplete the adult mouse brain from norepinephrine (also called noradrenaline), this neurotransmitter was added as a physiological negative regulator of the adult periventricular neural stem cell niche to the orchestra of humoral factors controlling adult neurogenesis. This knowledge might ultimately lead to new therapeutic approaches to influence neurogenesis in hypothalamus-related metabolic diseases or to stimulate endogenous regenerative potential in neurodegenerative processes such as Parkinson's disease

2 | METHODS

2.1 | Animals

Nestin-GFP heterozygous mice,[18] Tis21-GFP heterozygous mice[19] as well as C57BL/6N mice (Charles River Laboratories, Sulzfeld; Germany) were kept in standard housing conditions. Studies on NE innervation of periventricular regions were performed on Nestin-GFP transgenic mice (expressing green fluorescent protein [GFP] under the promotor of the *Nestin* gene for labeling NPCs) as described in detail previously.[18] Tis21-GFP transgenic mice (expressing GFP under the promotor of the *Tis21* gene also known as *PC3* gene) were used for visualization of NPCs starting neuronal differentiation at their final mitosis in vitro as described formerly.[19-21] All other experiments were performed on C57BL/6N mice. All experiments were performed on 8 to 12 weeks old male mice carried out according to local and federal regulations regarding research with animals.

2.2 | HPLC assay of tissue norepinephrine content

Brain tissue from periventricular regions of the adult mouse brain (200 μm surrounding the ventricles) was microdissected and immediately frozen in liquid nitrogen. Tissue samples were thawed, weighed, and sonicated in 0.05 M perchloric acid for 60 seconds resulting in 10% (w/v) homogenate suspensions. These were centrifuged at 48 000*g* for 20 minutes at 4°C, and 50 μL samples of the supernatants injected directly into a high performance liquid chromatography (HPLC) system with electrochemical detection (Gynkotek GmbH, Germering, Germany) for analysis of tissue NE according to the method as previously reported.[22,23] The HPLC system consisted of an AGILENT 1100 series (Bio-Rad, Munich, Germany), a Nucleosil 120-5C18 reverse phase (250 × 4.6 mm) analytical column (Macherey-Nagel, Düren, Germany), and an electrochemical detector (model 1640; Bio-Rad). The signal from the detector was recorded and data analyses were performed using an AGILENT Chem Station for LC9D (Bio-Rad). Concentrations were calculated from the peak height with the aid of an external standard. The investigator was blind to the experimental condition.

2.3 | Intracerebroventricular infusion

For intracerebroventricular (i.c.v.) infusion, osmotic minipumps (flow rate 0.5 μL/h over 7 days infusion; Alzet, Cupertino, California) were filled with either α1-adrenoreceptor (AR) and β-AR antagonists (50 μM prazosin and 50 μM propranolol), 20 μM NE (all from Sigma-Aldrich, Darmstadt, Germany) or a combination of AR antagonist and NE dissolved in artificial cerebrospinal liquor (aCSF) containing 0.5%

ascorbic acid to ensure NE stability over time (Sigma-Aldrich). The cannula was implanted unilaterally into the left lateral ventricle at the following stereotaxic coordinates according to rat brain atlas in stereotaxic coordinates by Paxinos & Watson[24]: −0.5 mm antero-posterior (AP), 1.3 mm medio-lateral (ML) and 2.5 mm dorso-ventral (DV) to Bregma and dura, respectively, as described previously.[25] To check for stability of NE over the time period of infusion, 20 μM NE was dissolved in aCSF containing 0.5% ascorbic acid and incubated at 37°C for 7 days with subsequent determination of NE levels using HPLC analysis. We observed a high stability of NE under these simulated in vivo conditions after 7 days with a recovery rate of 96.4 ± 2.5% (n = 4). Norepinephrine content in vivo as measured by HPLC in microdissected tissue (200 μm surrounding the ventricles) revealed a 3.3-fold increase in NE content in SVZ after i.c.v. infusion of NE for 7 days (137 ± 25 ng/g tissue) compared to controls (41 ± 26 ng/g tissue; $P = .025$; unpaired two-sided t-test).

2.4 | Neurotoxic lesion with Dsp4

To study the effects of endogenous NE, norepinephrine neurons were deleted by high doses of the neurotoxin *N*-(2-Chloroethyl)-*N*-ethyl-2-bromobenzylamine hydrochloride (Dsp4) in combination with the serotonin transporter inhibitor fluoxetine to protect serotoninergic neurons from degenerating and thereby achieving selective NE neuron depletion.[26] Mice received two intraperitoneal injections (1 week apart) of 50 mg/kg of Dsp4 30 minutes after a single i.p. injection of 10 mg/kg fluoxetine (all from Sigma-Aldrich). The neurotoxic lesion was confirmed by immunohistochemistry of norepinephrine neurons showing a massive loss of norepinephrine neurons (>80%) without affecting serotoninergic neurons (Supplemental online Figure 3) as described previously.[26,27]

2.5 | Treatment with thymidine analogs

For in vivo labeling of proliferating cells, thymidine analogs incorporation was performed according to previous studies of Vega and Peterson.[28] In brief, mice received each day of i.c.v. infusion an intraperitoneal (i.p.) injection of 57.5 mg/kg bodyweight IdU (iododeoxyuridine; MP Biomedicals, Eschwege, Germany) as well as a single i.p. injection of 42.5 mg/kg bodyweight CldU (chlorodeoxyuridine; Sigma-Aldrich) dissolved in sterile 0.9% NaCl solution.

2.6 | Tissue processing and immunohistochemistry

For immunohistochemical analysis mice were anesthetized with ketamine (50 mg/kg bodyweight) and transcardially perfused with 0.9% NaCl followed by 4% ice-cold paraformaldehyde (PFA, Sigma-Aldrich) in PBS. Brains were removed and post-fixed overnight in 4% PFA and subsequently incubated with 30% sucrose. Coronal sections (40 μm thickness) were cut using a sliding microtome (SM 2010R, Leica, Nussloch, Germany). Free-floating cryo-sections were stained as described previously[3] using the following primary antibodies: mouse anti-BrdU, 1:500 mouse anti-Mash1 1:100 (both from BD Pharmingen, San Diego, California), sheep anti-BrdU 1:250, rabbit anti-Egf receptor 1:100, chicken anti-Gfap 1:1500 rabbit anti-Sox2 1:1000 (all from Abcam, Cambridge, UK), rat anti-BrdU 1:500 (Bio-Rad, München, Germany), goat anti-Dcx C18 1:100, goat anti-Vimentin 1:200 (both from Santa Cruz Biotechnology, Dallas, Texas), rabbit anti-Iba1 1:1000 (Wako Chemicals, Richmond, Virginia), mouse anti-NET 1:200 (Mab Technologies, Stone Mountain, Georgia), rabbit anti-NeuN 1:2000 (Millipore/Merck, Darmstadt, Germany), rabbit anti-NG2 1:400 (Chemicon, Temcula, California), rabbit anti-5 HTT 1:500 (ImmunoStar, Hudson, Wisconsin), sheep anti-Th 1:500 (Pel-Freez, Rogers, Arkansas).

2.7 | Quantitative real-time PCR

For in vivo mRNA expression analysis, adult mice were killed by decapitation and PGNs were micro-dissected. Dissected tissue was transferred into RNAlater solution (QIAGEN, Hilden, Germany). Isolation of total RNA was performed according the RNeasy Lipid Tissue Mini Kit (QIAGEN) guidelines and stored at −80°C until further use. For in vitro mRNA expression analysis, adult mice were killed by decapitation and NPCs were micro-dissected from the subventricular zone. NPCs were grown as neurospheres for 7 days under standard expansion conditions, as described previously[3,11] prior to isolating total RNA using the RNeasy Mini Kit (QIAGEN). RNA was stored at −80°C until further use. Quantitative real-time PCR (35 cycles and 58°C annealing temperature) was performed as one-step qRT PCR with Brilliant SYBR Green QRT-PCR Master Mix Kit (Stratagene, Sigma-Aldrich) with the primer pairs displayed in Supplemental online Table 1. Analysis of relative gene expression was performed using the $2^{-\Delta\Delta CT}$ method introduced by Livak and Schmittgen in 2008.[29]

2.8 | Tissue processing for in situ hybridization

Mouse brains were embedded in Tissue-Tek O.C.T. (Sigma-Aldrich) and frozen within ice-cold methylbutane. Coronal sections (20 μm thickness) were obtained using a cryostat. Thereafter, slides were briefly dried for 20 minutes at RT and post-fixed in 4% PFA for 30 minutes at 4°C. Finally, slides were washed with PBS, dehydrated in ethanol and subsequently dried. Sections were stored at −80°C until further use.

2.9 | PCR-derived antisense-RNA probes

As described previously,[30] PCR-fragments were produced under standard PCR conditions using Taq-Polymerase (35 cycles and 57°C annealing temperature). Plasmid-DNA (10 ng) containing the full-length cDNAs of ARs of interest served as PCR-templates. In each case, the gene-specific downstream-primer contained an artificially introduced T3-promoter at its 5′-end to enable synthesis of antisense transcripts. Primer sequences are as shown in Supplemental online Table 2.

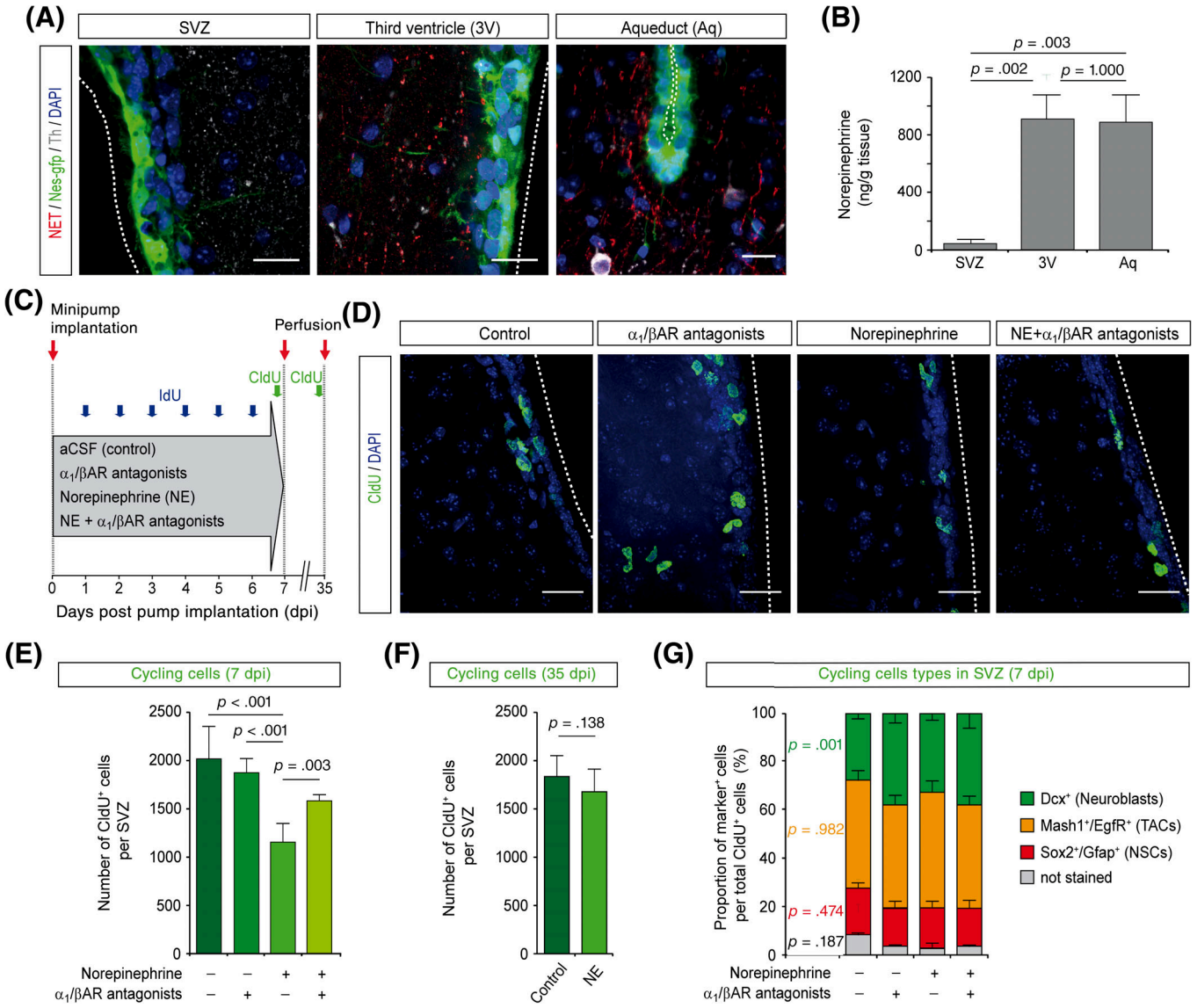

FIGURE 1 Exogenous norepinephrine impairs adult SVZ neuroprogenitor proliferation without major changes in the subtype composition within the SVZ. A, Norepinephrine (NE) innervation of the SVZ (right), third ventricle (3V, middle) and periaqueductal midbrain (Aq, left) periventricular germinal niches (PGNs). Staining of NE transporter (NET, red) and tyrosine hydroxylase (Th, grey) as markers for noradrenergic innervation showed minor innervation of the SVZ, contrary to high innervation of the hypothalamus and midbrain in close proximity to Nestin$^+$ neural progenitor cells (Nestin-GFP, green) in the PGN of the 3V and Aq. Cell nuclei were counterstained with DAPI. Scale bar = 25 μm. B, NE content as measured by HPLC in microdissected tissue (200 μm bordering the ventricle) verified histochemical observations and revealed significantly less endogenous NE in the SVZ compared to the hypothalamic 3V-PGN and midbrain Aq-PGN (*F*-value: 24.98; *P* = .001; one-way ANOVA). P-values are from Bonferroni post hoc *t*-tests (n = 3). C, Schematic representation of experimental paradigm to investigate NE effects on SVZ neurogenesis using intracerebroventricular (i.c.v.) infusions via osmotic minipumps. Artificial cerebrospinal fluid (aCSF, control), NE, α1/β adrenoreceptor (AR) antagonists as well as the combined NE/AR antagonist cocktail were infused for 7 days unilaterally into the lateral ventricle of adult mice along with various thymidine analogue application paradigms with either infusion-contemporaneous IdU or single-shot CldU administration at the end of the i.c.v. infusion (7 days after pump implantation, dpi). In some animals, CldU was applied 28 after termination of i.c.v infusion (35 dpi) to investigate the reversibility of NE effects. Mice were analyzed acutely (7 dpi) or after long-term period (35 dpi). D,E, Effects of NE and AR antagonists on currently proliferating CldU$^+$ cells (green) within the adult SVZ at 7 dpi. The inhibitory effect of exogenous NE on SVZ cell proliferation were completely reversed mediated by co-administration of α1/βAR antagonists (*F*-value: 18.62; *P* < .001; one-way ANOVA). *P*-values are from Bonferroni post hoc *t*-tests (*n* = 7-8). Cell nuclei were counterstained with DAPI (blue). Scale bars = 25 μm. F, Quantitative analysis of currently proliferating cells in the SVZ at 35 dpi (28 days after termination of NE excess) revealed complete reversal of NE-induced inhibition of SVZ cell proliferation and no differences to i.c.v. infusion of aCSF (unpaired two-sided *t*-test, *n* = 10-11). G, Quantitative characterization of actively proliferating SVZ cell lineages, namely neuroblasts, transient amplifying cells (TACs) and neural stem cells (NSCs). Solely the mean proportions of proliferating neuroblasts (Dcx$^+$/CldU$^+$) showed significant differences between the animal groups (*P*-values are from one-way ANOVA; *n* = 5-8). SVZ, subventricular zone

2.10 | In vitro transcription of RNA probes

For generation of digoxygenin (DIG)-labeled RNA probes, in vitro transcription was performed. Ten microliters of transcription solution, comprising 0.5 μL linearized template DNA, 2 μL transcription puffer (5×), 1 μL DIG-RNA labeling mixture (10×), 1 μL RNase inhibitor (20 U/μL) and 1 μL RNA polymerase (T3), was incubated 2 hours at 37°C. Subsequently, template DNA was removed by an additional incubation step of RNase-free DNase solution for 15 minutes and finally RNA polymerase activity was stopped by EDTA (0.2 M, pH 8.0). Thereafter, RNA probes were separated using LiCl and ethanol by incubating 2 hours at −70°C prior to an additional incubation step over night at −20°C. Next day, RNA probes were spun down at 14 000 rpm for 10 minutes at 4°C, washed in ethanol one time prior to an additional centrifugation step. Subsequently, electrophoresis was taken in advantage to confirm successful in vitro transcription of RNA probes of interest. Finally, RNA probes were dissolved in DEPC pretreated water and stored at −80°C until further use.

2.11 | In situ hybridization

In brief, cryo-sections were briefly dried for 20 minutes at RT, slides were treated three times with 3% hydrogen peroxide and washed in PBS, to block endogenous activity of alkaline phosphatase. Subsequently, sections were permeabilized by pretreatment with proteinase-K buffer for 10 minutes at 37°C prior to incubation in proteinase-K (10 μL/mL proteinase-K buffer) for 20 minutes at 37°C. In addition, sections were post-fixed with 4% PFA and washed three times in PBS. Next, sections were pre-hybridized in hybridization solution for 2 hours at 65°C prior to overnight incubation in hybridization solution containing RNA probes of interest. The following day, slides were washed several times in SCC buffer (descending order of salt content) at 65°C, followed by an additional washing step in DIG I buffer for 10 minutes at RT. Thereafter, sections were incubated in blocking solution, containing 1% milk powder in DIG I buffer, for 30 minutes at RT and anti-DIG antibody was administered in blocking solution for 3 hours at RT. Finally, slides were washed two times in DIG I as well as once in DIG II buffer prior to incubation in NBT/BCIP substrate solution. After washing in TE buffer and dd H_2O, sections were mounted in Mowiol 4-88 (Sigma-Aldrich) and stored at 4°C until confocal imaging.

2.12 | Adrenoceptor antagonist and norepinephrine treatment in vitro

Adult mice were killed by decapitation and NPCs were micro-dissected from the SVZ. Primary neurospheres were kept under expansion condition for 7 days (Neural Basal Medium (GIBCO, Dublin, Ireland) containing 2% B27 (Sigma-Aldrich), 1% Glutamax (GIBCO), 1% Penicillin/Streptomycin (Sigma-Aldrich) additionally treated with 1:1000 Egf and 1:1000 Fgf2 (both from Sigma-Aldrich). Secondary neurospheres were treated with either 1 mM ascorbic acid, α1-AR and β-AR antagonists (1 μM prazosin and 1 μM propranolol), 1 μM NE (all from Sigma-Aldrich) or a combination of AR antagonists and NE for the following 5 days. 1 mM ascorbic acid was used to avoid oxidation of NE.

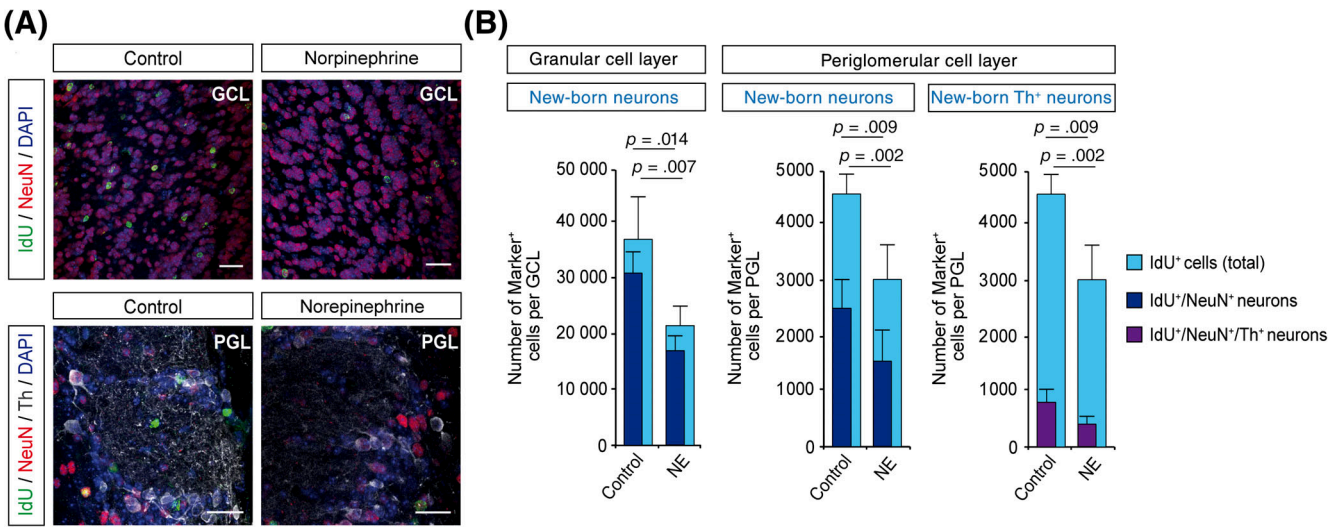

FIGURE 2 Exogenous norepinephrine impairs adult neurogenesis in the olfactory bulb. A, Representative images showing new-born neurons within the olfactory bulb at 35 dpi. Upper panel shows new-born neurons (stained for proliferation marker IdU [green] and the neuronal marker NeuN [red]) within the glomerular cell layer (GCL), while lower panel displays new-born dopamine neurons (additionally stained for catecholaminergic marker Th [grey]) within the periglomerular cell layer (PGL) generated under either control or NE administration. Cell nuclei were counterstained with DAPI (blue). Scale bars = 25 μm. B, Quantitative evaluations of total cells that were in cell cycle 28 days before (IdU^+, bright blue bars) and new-born neurons in the GCL and PGL (co-expressing NeuN [dark blue] or NeuN and Th [purple bars]) after control and NE i.c.v. infusion. *P*-values are from unpaired two-sided *t*-tests (n = 4-5). All data are expressed as mean ± SEM. NE, norepinephrine

2.13 | Immunocytochemistry

For labeling of proliferating cells in SVZ-derived sphere-forming cell colonies, a standard protocol was used as described previously.[3] In brief, Accustain-fixed neurospheres were stained using rabbit anti-Ki67 1:500 (Leica Biosystems), mouse anti-Tuj1 1:500 (Covance) and fluorescence labeled secondary antibodies (Alexa Fluor, Invitrogen, Carlsbad, CA). Cell nuclei were counterstained with Hoechst 33342 (Invitrogen).

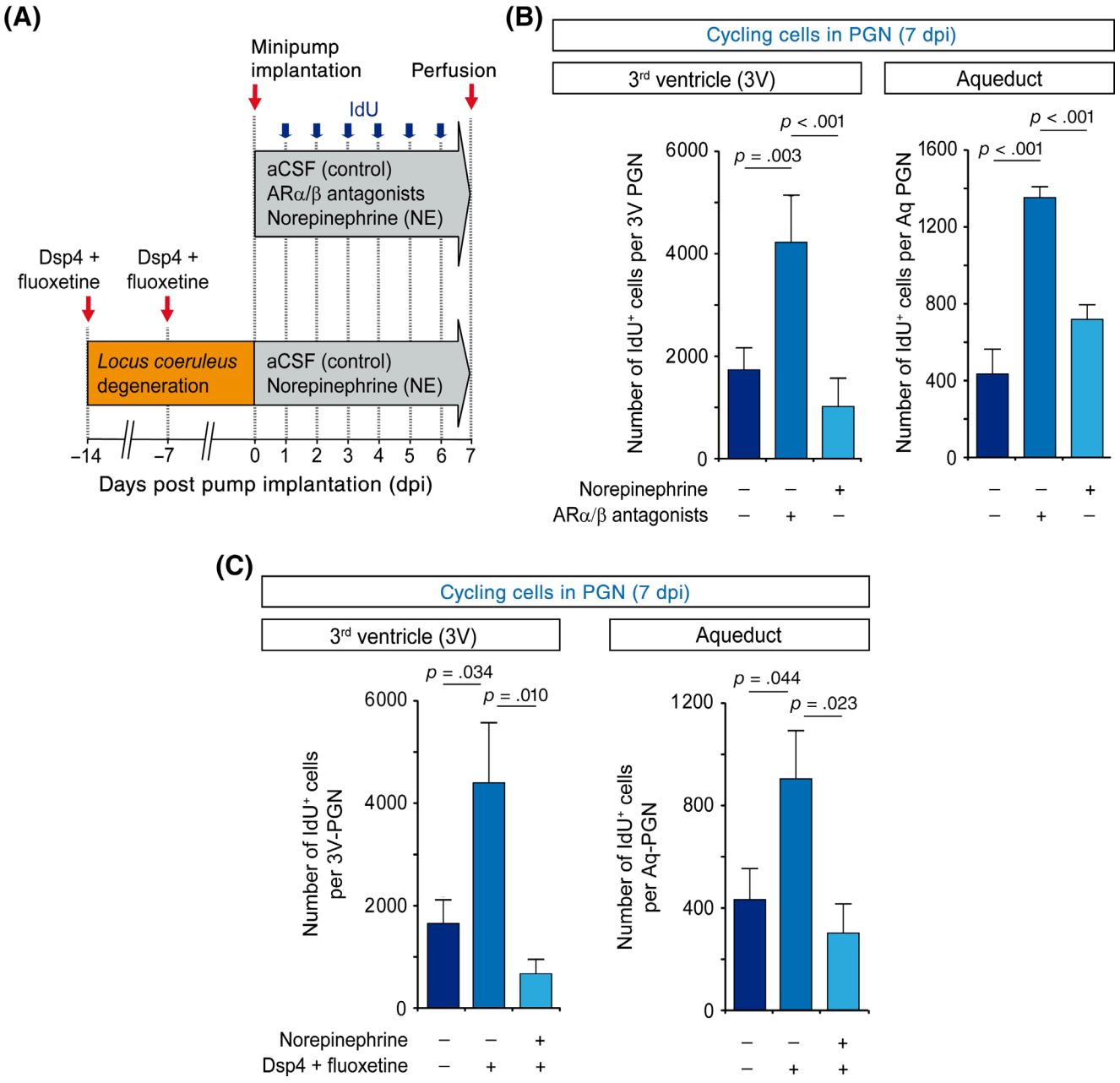

FIGURE 3 Depletion of endogenous norepinephrine signaling induces neuroprogenitor cell proliferation within the hypothalamic and midbrain neurogenic niches. A, Schematic representation illustrating experimental paradigms to deplete NE signaling by pharmacological (transient i.c.v. infusion of α1/βAR antagonists) and toxic approaches (permanent depletion of NE neurons by *N*-(2-Chloroethyl)-*N*-ethyl-2-bromobenzylamine hydrochloride (Dsp4) treatment). Events of adult neurogenesis within the hypothalamic and midbrain PGNs were analyzed at 7 dpi. B,C, Quantification of the effects of pharmacological inhibition of NE signaling by α1/β AR antagonists, B, and toxic NE neuron depletion by Dsp4 treatment, C, on cell proliferation in the hypothalamic (3V-PGN) and midbrain (Aq-PGN) niche as measured by IdU incorporation. One-way ANOVA revealed significant differences between the animal groups for both approaches (*F*-value: 29.91; *P* < .001 [3V-PGN] and *F*-value: 32.61; *P* < .001 [Aq-PGN] for pharmacological, B; *F*-value: 12.17; *P* = .008 [3V-PGN] and *F*-value: 9.45; *P* = .013 [Aq-PGN] for toxic approach, C). Depicted *P*-values are from Bonferroni post hoc *t*-tests (*n* = 3-5). PGN, periventricular germinal niche

2.14 | Cell counts and statistical analysis

For absolute cell counts, series of every sixth section (240 μm apart) were stained for IdU or CldU. Positive cells were counted throughout the rostro-caudal extent of either the SVZ, the periventricular germinal niche of the hypothalamus bordering the third ventricle (3V-PGN), the midbrain bordering the aqueduct (Aq-PGN) or subgranular zone of the dendate gyrus (SGZ) of the hippocampus using the optical fractionator method of the StereoInvestigator 7.50.4 software (MBF Bioscience, Williston, Vermont). The resulting numbers were multiplied by 6 to obtain the estimated total number of labeled cells. For proportional analyses of the cell phenotype, 100 cells per region were analyzed for any combination of cell phenotype using the confocal microscope (Leica SP5, 40× oil objective) in sequential scanning mode for the co-expression of different markers. Double labeling was confirmed by *z*-series of the entire nucleus or cell in question.

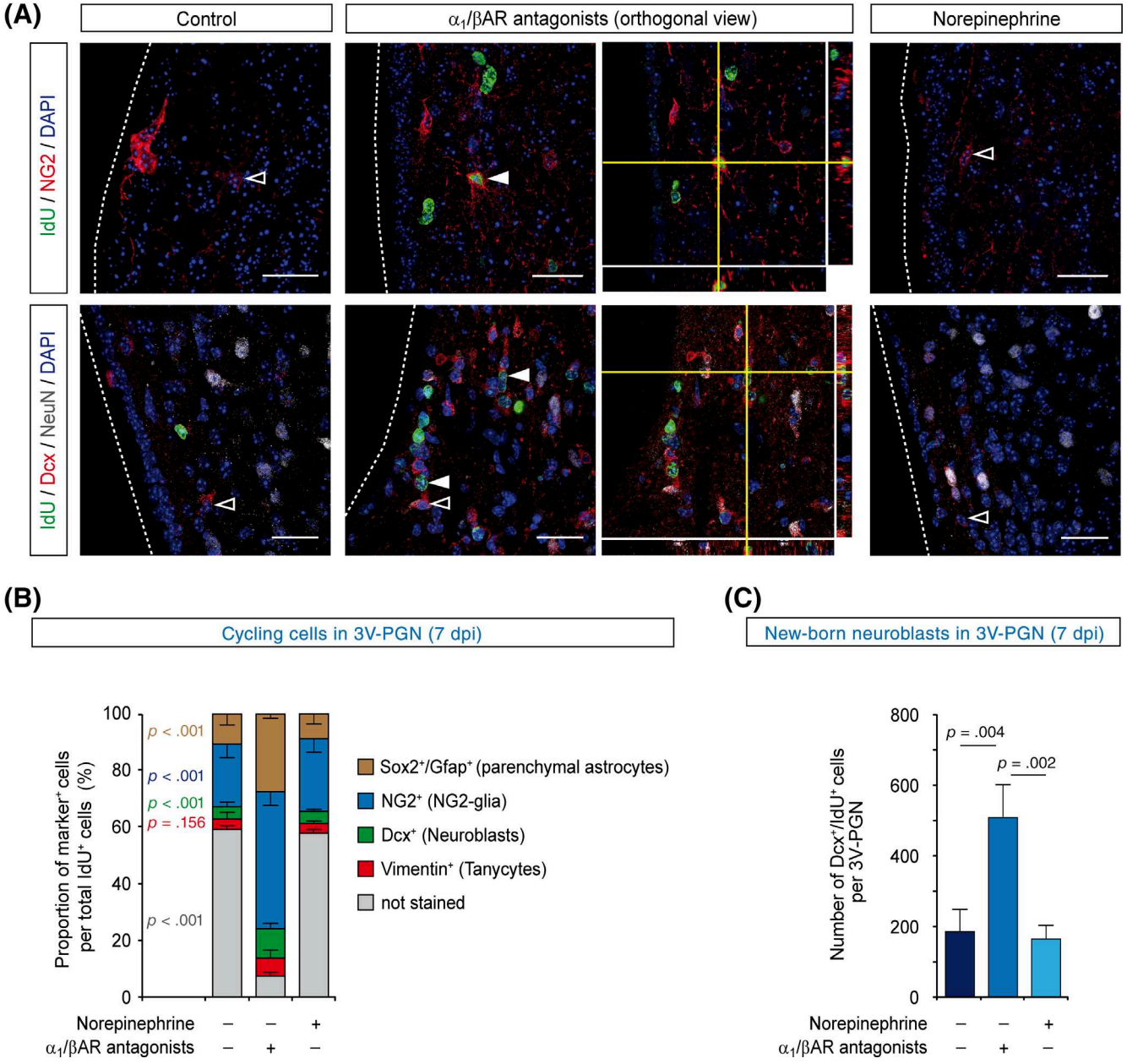

FIGURE 4 Phenotyping of cell type composition within the hypothalamic PGN revealed enhanced adult neurogenesis by blocking endogenous norepinephrine signaling. A, Representative images showing proliferative NG2$^+$ glia (upper panel) and Dcx$^+$ neuroblasts (lower panel) within the 3V-PGN. Cell nuclei were counterstained with DAPI (blue). White closed arrowheads indicate proliferating cell populations during their S phase, whereas open arrowheads point non-DNA replicating marker$^+$ cells. Scale bars = 25 μm. B, Quantification of the proliferative cell types. *P*-values are from one-way ANOVA (*n* = 3-5). C, Quantification of the effects of pharmacological inhibition of NE signaling by α1/βAR antagonists on hypothalamic new-born neuroblasts. One-way ANOVA revealed significant differences between the animal groups (*F*-value: 19.29; *P* = .001; *n* = 3-5). Depicted *P*-values are from Bonferroni post hoc *t*-tests. All data are expressed as mean ± SEM. PGN, periventricular germinal niche

We determined mean differences between groups using two-sided unpaired *t*-test, or one-way/two-way analysis of variance (ANOVA) for multiple comparisons and post hoc *t*-tests with Bonferroni adjustment as appropriate. The data met the assumption of these tests with normal distribution of data (Kolmogorow-Smirnow test and/or visual check of box plots) and equal variances (Levene's test for homogeneity of variances). We conducted all statistical analyses using the SPSS software (Version 23). Number of animals/conditions and specific statistical analyses used in each experiments are indicated in the figure legends and/or text. All data are presented as mean of at least three individual experiments ± SE (SEM).

3 | RESULTS

3.1 | Norepinephrine innervation of the adult neural stem cell niches negatively correlates with neuroprogenitor cell proliferation

We initially investigated NE innervation of the SVZ, the hypothalamic 3V-PGN and the midbrain periaqueductal Aq-PGN. The SVZ showed only marginal expression of the NE transporter (NET) as a marker for noradrenergic varicosities,[31] and consistently very low levels of NE, while the tissue in close proximity to the 3V-PGN expressed high levels of NET protein and the 3V-PGN itself contained high concentrations of NE (Figure 1A,B). We did not detect adrenaline in any PGN by HPLC combined with electrochemical detection. In contrast, all PGNs displayed similar expression patterns of adrenoceptors (ARs) with high levels of Adrα1b, Adrβ1, and Adrβ3 (Supplemental online Figure 1a,b). Although our previous data show that 3V-PGN and Aq-PGN do not contain relevant amounts of synaptic structures,[3] these findings are in agreement with the concept of non-junctional NE release over a broad diffusion zone including the PGNs.[16] Together, the pattern of NE innervation of the adult periventricular neurogenic niches is inversely associated with their NPC proliferation capacity.

3.2 | Norepinephrine reversely inhibits SVZ cell proliferation and reduces olfactory bulb neurogenesis

To test whether NE regulates NPC proliferation within the SVZ, we traced proliferating cells using one-stage CldU administration after artificial increase of NE levels in the SVZ by intraventricular NE infusion for seven days (Figure 1C): Intriguingly, exogenous NE inhibited cell proliferation within the SVZ via α_1/βAR signaling (Figure 1D,E). These effects were reversible and not detectable anymore at 28 days after the termination of NE excess (Figure 1C,F). We then examined cell type distribution of the proliferating cell population with respect to NE activation: No relevant differences could be observed due to exogenous NE administration in the distribution of $Sox2^+$/$Gfap^+$ NSCs, transit-amplifying cells expressing Mash1 and the EGF receptor, and Dcx^+ neuroblasts (Figure 1G, Supplemental online Figure 2). Contrarily, application of AR antagonists provoked a small increase of cycling neuroblasts when compared to control condition (Figure 1G, Supplemental online Figure 2), suggesting minor effects of the low levels of endogenous NE on neuroblasts within the SVZ, which did not translate into changes of the overall proliferating NPC pool.

To determine whether the NE-induced inhibition of NPC proliferation within the SVZ translates into changes of net neurogenesis in the olfactory bulb as the final destination of SVZ neuroblasts,[32] we

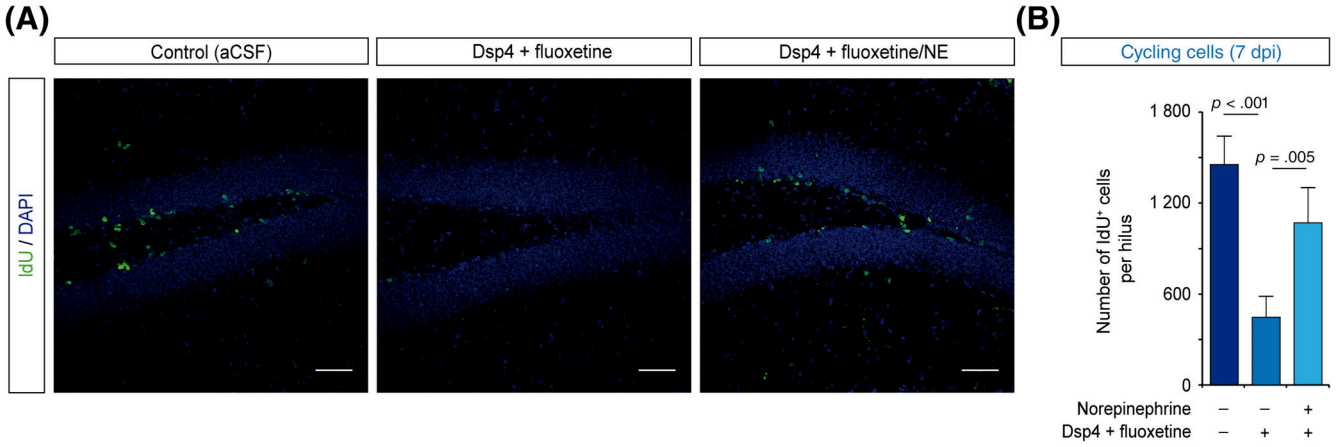

FIGURE 5 Degeneration of *Locus coeruleus* NE neurons inhibits cell proliferation in the dentate gyrus of the hippocampus through NE depletion. Hippocampal neurogenesis was analyzed in the same animals used for the studies on periventricular neurogenesis as displayed in Figure 3 to conclusively demonstrate the opposite effects of NE on periventricular vs hippocampal NPC proliferation. A, Immunofluorescent staining of the dentate gyrus showing decreased number of proliferating IdU^+ cells by permanent NE depletion using *N*-(2-Chloroethyl)-*N*-ethyl-2-bromobenzylamine hydrochloride (Dsp4) neurotoxic treatment. Supplementation of NE by intraventricular infusion normalized proliferation in the dentate gyrus confirming NE dependency of Dsp4 actions on hippocampal neurogenesis. Scale bars = 25 μm. B, Quantification of total proliferating IdU^+ cells per dentate gyrus (one-way ANOVA: *F*-value: 23.34; $P < .001$; $n = 3$-5). *P*-values are from Bonferroni post hoc *t*-tests. NE, norepinephrine; NPC, neuroprogenitor cell

marked proliferating cells with repetitive IdU administration during intraventricular NE infusion and quantified SVZ-derived new-born neurons 28 days later (Figure 1C). NE infusion significantly reduced not only the numbers of the total IdU$^+$ cell population representing all cells newly born during the NE administration time period within both the olfactory granular cell layer (GCL) and periglomerular cell layer (PGL), but also the numbers of new-born NeuN$^+$ interneurons including their Th$^+$ dopaminergic subtype (Figure 2A,B). The proportion of

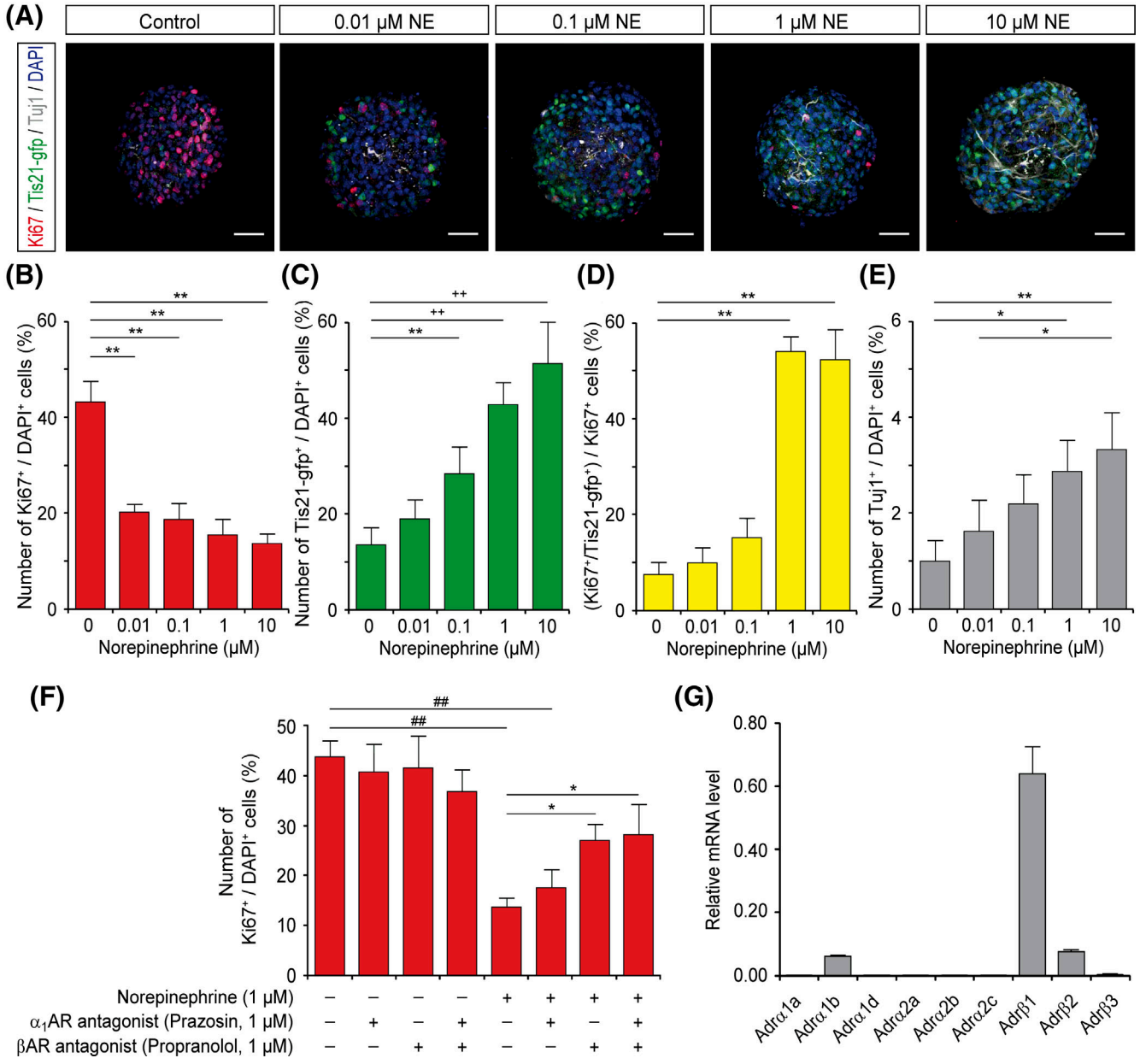

FIGURE 6 Norepinephrine inhibits proliferation and promotes neuronal differentiation of adult SVZ progenitor cells in vitro. A, Representative confocal images of transgenic Tis21-GFP (green) SVZ-derived adult neurospheres stained with DAPI (blue), Ki67 (red) and Tuj1 (grey) under control condition and various NE concentrations ranging from 0.01 to 10 μM. Scale bars = 25 μm. B-E, Numbers of proliferating Ki67$^+$ cells (B), Tis21-GFP$^+$ cells relative to total cells numbers (C), cells in last cell cycle (Ki67$^+$/Tis21-GFP$^+$ cells) expressed as a percentage of total proliferating Ki67$^+$ cell numbers (D), and numbers of Tuj1$^+$ neurons relative to total cell numbers with respect to NE treatment. One-way ANOVA revealed significant differences for all parameters tested (F-value: 61.54; $P < .001$ for Ki67$^+$ cells, F-value: 48.97; $P < .001$ for Tis21-GFP$^+$ cells, F-value: 47.48; $P < .001$ for cells in last cell cycle, and F-value: 7.03; $P < .001$ for Tuj1$^+$ neurons; n = 4-6). F, Pharmacology of NE effects on adult NPC proliferation. Proliferative Ki67$^+$ cells are expressed as a percentage of total cell number (DAPI$^+$ cells). One-way ANOVA revealed significant differences between treatments (F-value: 7.90; $P = .001$ for Ki67$^+$ cells; n = 5-6). G, Expression of the various ARs in adult NPC cultures as measured on mRNA level by qRT-PCR. Data are mean values ± SEM of at least three independent experiments. * indicates $P < .05$, ** indicates $P < .01$, $^{++}$ indicate $P < .01$ when compared to 0, 0.01 and 0.1 μM norepinephrine, $^{\#\#}$ indicate $P < .01$ when compared to all 0 μM NE conditions (unpaired two-sided t-tests with Bonferroni adjustment). NE, norepinephrine; NPC, neuroprogenitor cell; SVZ, subventricular zone

newborn neurons to the total IdU^+ cell population was not altered by NE administration within the two regions of the olfactory bulb (GCL: 83.4 ± 4.8% under control vs 87.0 ± 3.9% under NE conditions [P = .191]; PGL: 55.6 ± 7.9% vs 51.5 ± 10.7% [P = .496]), again showing that NE exerts its influence on NPC proliferation rather than on NPC differentiation or cell fate decisions.

3.3 | Blocking norepinephrine actions activates adult hypothalamic and midbrain neuroprogenitor proliferation and early neurogenesis events

The anti-proliferative effects of exogenous NE within the SVZ prompted us to investigate whether the high endogenous NE levels within more caudal PGNs such as that of the hypothalamic 3V-PGN and midbrain Aq-PGN[2,3,6] might be restrictive for their NPC proliferation capacity. We therefore dissected the effects of AR antagonism on NPC proliferation within the 3V-PGN and Aq-PGN (Figure 3A). Intraventricular infusion of α_1/βAR antagonists to block endogenous NE actions led to a massive increase of IdU^+ proliferating cells within both PGNs after 7 days (Figure 3B). Administration of exogenous NE was not able to further elevate the high endogenous NE content (P = .920 for 3V-PGN and P = .984 for Aq-PGN) and consequently did not further inhibit cell proliferation (Figure 3B). For verification of the obtained results, we applied the high-dose Dsp4-based neurotoxic model allowing the selective degeneration of Th^+/NET^+ noradrenergic LC neurons projecting amongst other regions to the hypothalamus and the midbrain (Figure 3A, Supplemental online Figure 3).[26,33] The selective depletion of endogenous NE within the hypothalamus led to a 2.1- to 2.5-fold increase in PGN cell proliferation (Figure 3C). Intriguingly, parallel intracerebral infusion of exogenous NE completely reversed Dsp4 effects (Figure 3C) confirming that they are entirely mediated through NE signaling.

Phenotyping of the proliferating cell population revealed no changes of the proportion of ependymal $vimentin^+$ tanycytes by NE antagonism, but an increase of other hypothalamic NPC populations, namely $NG2^+$ glial cells, $Sox2^+/Gfap^+$ parenchymal astrocytes and Dcx^+ neuroblasts (Figure 4A,B, Supplemental online Figure 4). These relative changes translated into elevations of the total cell numbers of these cell types, in particular of newly generated Dcx^+/IdU^+ neuroblasts within the 3V-PGN (Figure 4C). Consistently, we found NE-mediated NPC proliferation mainly in the subependymal parenchyma adjusted to the lateral wall of the 3V in proximity to the dorsomedial and ventromedial hypothalamus and the arcuate nucleus (Figure 4A, Supplemental online Figure 4), but not at the median eminence bordering the medio-basal part of the 3V (data not shown).

3.4 | Impairment of norepinephrine innervation decreases adult hippocampal neurogenesis

Since NE is reported to execute stimulatory action on NPC proliferation within the subgranular zone of the dentate gyrus (SGZ) within the adult hippocampus,[14,15,26] we tested the effects of NE depletion on hippocampal NPC proliferation in Dsp4-treated mice. The selective depletion of endogenous NE led to a dramatic decrease of SGZ cell proliferation (Figure 5). Intriguingly, parallel intracerebral infusion of exogenous NE completely reversed Dsp4 effects confirming that they are entirely mediated through NE depletion.

3.5 | Norepinephrine suppresses neuroprogenitor proliferation and promotes neurogenesis through β-adrenoceptor signaling

In subsequent in vitro experiments we confirmed the inhibitory NE effects on adult NPC proliferation isolated from the SVZ and grown in neurosphere cultures.[3,11] NE dose-dependently suppressed NPC proliferation in vitro and promoted their cell cycle exit and subsequent neuronal differentiation (Figure 6A-E). Pharmacological characterization of NE actions revealed that its anti-proliferative effects are mediated through β-AR signaling (Figure 6F,G).

4 | DISCUSSION

Here we add NE as a physiological negative regulator of the adult periventricular neural stem cell niche of the mammalian brain to the orchestra of humoral factors controlling adult periventricular neurogenesis.[12,13] Its regulatory role is particularly characterized by anti-proliferative effects on NPCs most likely mediated through direct pharmacological stimulation of β-AR signaling. Since previous studies used pharmacological agents to alter AR signaling, such as antidepressants or small molecule AR inhibitors, the effects of the endogenous compound NE were previously undiscovered. This is, however, of particular importance in the adrenergic system, because the AR system is extremely diverse and includes various signaling pathways leading to several physiologic actions including opposite effects on adult hippocampal neurogenesis.[34] Indeed, our data show - to our knowledge as the first case in adult neurogenesis regulation - that NE exerts contrary effects on adult neurogenesis depending on the neurogenic niche with inhibitory effects within the PGNs but stimulating effects in the dentate gyrus (SGZ) of the hippocampus.

Adult neurogenesis within the SVZ is regulated by various mechanisms, which include not only factors from the immediate niche and the adjacent cerebrospinal fluid, but also signals from neurotransmitters released from neurons residing outside the niche and coupling brain activity to SVZ neurogenesis.[12,13,35,36] These neuronal inputs particularly comprise of serotoninergic projections from the dorsal raphe nucleus as well as striatal glutamatergic/GABAergic and midbrain dopaminergic projections.[12,13,35-42] These inputs promotes either quiescence and/or proliferation of distinct NPC populations within the SVZ.[12,35-42] Due to its particular innervation pattern of the adult brain, the NEergic system is a candidate negative regulator of NPC performance within the caudal PGNs including the hypothalamic neurogenic niche: The LC as the major NEergic system projects via non-junctional varicosities to almost all major brain regions including hypothalamus, (periaqueductal)

midbrain, and hippocampus.[16,17,33,43,44] The only major region that does not receive input from the LC are the basal ganglia including the striatum.[33,43,44] By performing histological and neurochemical analyses of the PGNs, we show here that the pattern of NE innervation of the adult PGNs is inversely associated with their NPC proliferation capacity with low NE levels and high NPC proliferation capacity in the SVZ but high NE levels and low NPC proliferation capacity in the caudal PGNs. Using exogenous NE through i.c.v. infusion combined with pharmacological AR inhibition we demonstrate that NE reversely inhibits SVZ cell proliferation and subsequently reduces olfactory bulb neurogenesis with minor or no effects on cell fate decision. Subsequent cell cultures studies confirmed inhibitory NE action on adult SVZ NPC proliferation with dose-dependent suppression of NPC proliferation by promoting their cell cycle exit and subsequent neuronal differentiation most likely through direct β-AR signaling.

The most apparent conclusion from the anti-proliferative effects of exogenous NE within the SVZ is that the high endogenous NE levels in all other PGNs might be a restrictive factor of NPC proliferation and thus neurogenesis in more caudal periventricular regions such as that of the hypothalamic 3V-PGN and midbrain Aq-PGN.[2,3,6] Indeed, pharmacological blockade of NE actions activates adult NPC proliferation and early neurogenesis events in hypothalamic and midbrain PGNs. Toxic degradation of NE neurons within the LC by the high-dose Dsp4 application confirms the results from the pharmacological studies. Since there is a debate on the selectivity of the Dsp4 toxicity for the NE system,[45] we added the serotonin transporter inhibitor fluoxetine to the protocol to enhance selectivity for NE neurons by protecting serotoninergic neurons.[27] Histology studies indeed certify the strong and selective degradation of the NE systems (Supplemental online Figure 3). Another potential caveat for the Dsp4 treatment experiment is the possible initial increase in NE release by the neurotoxin.[46] Even more important, thus, is the complete rescue of Dsp4-fluoxetine effects by administration of exogenous NE through i.c.v. infusion furthermore confirming that the effects of Dsp4-induced degradation on NPC proliferation are really mediated through NE depletion in our model.

Deep phenotyping of the proliferating cell population revealed no changes of the proportion of ependymal vimentin$^+$ tanycytes the 3V by NE antagonism, but an increase of other hypothalamic NPC populations, namely NG2$^+$ glial cells, Sox2$^+$/Gfap$^+$ parenchymal astrocytes and Dcx$^+$ neuroblasts translating into an elevation of newly generated Dcx$^+$/IdU$^+$ neuroblasts. Consistently, we found NE-mediated NPC proliferation mainly at the subventricular/subependymal parenchyma directly adjacent to the tanycyte layer of the lateral wall of the 3V in proximity to the dorsomedial and ventromedial hypothalamus and the arcuate nucleus (Figure 4A, Supplemental online Figure 4), but not at the median eminence bordering the medio-basal part of the 3V. Several previous studies have reported neurogenesis to occur not only in the ventricular wall of the 3V but also in the hypothalamic parenchyma, although here the evidence is much more controversial.[47] This parenchymal hypothalamic neurogenesis has been reported to be regulated by several cytokines or neurotrophic factors as well as by specific neurodegenerative events and dietary signals.[47,48] The relationship between these parenchymal hypothalamic NPCs and those of the hypothalamic ventricular zone remains unclear, but might be similar to other brain regions with ventricular NPCs (tanycytes in the hypothalamus) might give rise to parenchymal NPCs.[49,50] However, our results provide strong evidence that NE acts as an endogenous negative regulator of the hypothalamic periventricular stem cell niche, with no or only minor effects on ependymal tanycytes.[8,51] Future studies are warranted to finally define the sequence of NPC subtypes involved in NE-stimulated hypothalamic neurogenesis.

The hippocampal neurogenic niche within the SGZ of the dentate gyrus receives dense innervation from the LC noradrenergic system.[52-54] Early approaches to assess the effects of NE on adult hippocampal function including neurogenesis used pharmacological agents to increase NE levels in the hippocampus, such as antidepressants, or various AR agonists or antagonists. Chronic antidepressant treatment increases neurogenesis in the hippocampus most likely through NE signaling,[14] but effects through other neurotransmitter systems, such as serotonin, have already been described in detail.[55,56] However, subsequent studies using AR agonists and antagonists showed complex actions of the various AR systems: Stimulation of α2-ARs decreases while stimulation of β-AR enhances hippocampal NPC proliferation and neurogenesis.[34,57,58] Although the β-AR subtype selectivity of these effects remained unclear from the animal studies, cell cultures studies on adult hippocampal NPCs strongly suggest that NE regulation of adult hippocampal neurogenesis is mediated through β2-AR and/or β3-AR subtype.[58,59] These data strongly show the necessity of studies addressing the direct actions of the endogenous compound NE (or its depletion) to investigate the net effect of NE on adult hippocampal neurogenesis. Indeed, more recent reports with long-term toxic depletion of LC NE neurons using the toxins anti-dopamine-β-hydroxylase (anti-DBH)-saporin[60] or Dsp4[26] with strong reduction of NE innervation of the hippocampus demonstrate that NE depletion leads to a fast reduction of NPC proliferation after a few days but no change of long-term survival and differentiation of SGZ NPCs after 4 to 9 weeks.[26,61,62] Although the adult hippocampal neurogenic niche was not in the focus of our studies, we analyzed also the effects of NE depletion on SGZ NPC proliferation in direct comparison to the effects of NE depletion using Dsp4-fluoxetine administration in the PGNs in the same animals. In agreement with the previous studies, we here show that NE depletion provokes reduction in SGZ progenitor proliferation. Although there is a debate on the selectivity of the Dsp4-fluoxetine protocol for the NE system (see above), the complete rescue of Dsp4-fluoxetine effects by administration of exogenous NE again confirms that the effects are indeed mediated through NE depletion. Together, NE is a stimulator of NPC proliferation within the SGZ of the dentate gyrus as the initial neurogenesis event but does not alter net survival or neuronal differentiation.

Since the physiological role of hypothalamic neurogenesis still remains to be determined in detail, future studies need to dissect the functional role of NE-mediated suppression of NPC proliferation and neurogenesis in the PGNs. However, the physiological role of NPC proliferation within the hypothalamic stem cell niche in long-term

body weight homeostasis including control of feeding and energy balance is intensively discussed in the recent literature.[8,47,48,51,63] In this concept, proliferation of NPCs—mainly NSCs located within the lateral hypothalamus, which are inhibited by NE signaling in our studies—is associated with body weight loss or maintenance, while proliferation of medio-basal NPCs appears to be associated with weight gain. The actions of NE on periventricular neurogenesis are in contrast to its effects on hippocampal neurogenesis with its stimulatory role on NPC proliferation within the SGZ of the dentate gyrus (our study and those of others[14,15]). Such differential or even opposite effects of one humoral factor on the two major neurogenic niches of the adult mammalian brain have not been reported yet for other factors, though the role of the other catecholaminergic neurotransmitter, that is, dopamine, in the regulation of adult neurogenesis is also in part controversial.[56,61,64] Several lines of evidence suggest that reduced adult hippocampal neurogenesis is associated with depression and consistently the behavioral effects of noradrenergic antidepressants in animal models depend on the generation of new neurons within the hippocampus.[14,65] Our data might thus indicate a mechanistic link between body weight homeostasis and depression[66] via the opposite effects of NE released by LC neurons on PGN vs hippocampal neurogenesis, and provide an explanation for long-term body weight gain by centrally acting noradrenergic compounds such as various antidepressants.[67,68] However, further investigations are warranted to completely dissect the physiological role of noradrenergic regulation of hypothalamic NPC proliferation and neurogenesis.

Although neurogenesis or dopaminergic neurogenesis in the midbrain region in response to toxic/disease insults or pharmacological interventions are still not convincingly proven,[4,64,69] the existence of functional NPCs with neurogenic and even dopaminergic differentiation potential in vitro has been demonstrated in the midbrain PGN.[11,64,70] Moreover, when these midbrain NPCs were transplanted into the neurogenic niche of the hippocampus, they also expressed neurogenic differentiation potential.[70] Intriguingly, NPCs from classical neurogenic niche such as the hippocampus did not show any neurogenic potential after transplantation into the adult midbrain.[70] Together, these data strongly implies cell-extrinsic factors restricting NPC proliferation and differentiation in the midbrain PGN. We provide here first evidence that NE might be one endogenous cell-extrinsic factor inhibiting NPC proliferation and subsequent neurogenesis in PGNs of the ventricular system caudal of the lateral ventricles.

5 | CONCLUSION

In conclusion, we demonstrate that endogenous norepinephrine inhibits periventricular neuroprogenitor cell proliferation and neurogenesis in the adult mammalian brain. We thus add norepinephrine to the orchestra of endogenous humoral factors regulating adult periventricular neurogenesis. Our data further indicate that norepinephrine has opposite effects on the two fundamental neurogenic niches of the adult brain: Its role is particularly characterized by antiproliferative effects on periventricular neuroprogenitors most likely mediated through direct stimulation of β-adrenoceptors, but stimulating effects on hippocampal neurogenesis. This knowledge not only helps to understand the principles of adult neurogenesis in general, but might ultimately lead to novel therapeutic approaches to influence neurogenesis in hypothalamus-related metabolic diseases such as obesity or type 2 diabetes[48,51] or to stimulate endogenous regenerative potential in neurodegenerative processes with focus in the midbrain region[4,64,69] such as in Parkinson's disease.

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

G.W.: designed, analyzed and performed all of the experiments, and wrote the manuscript; M.D.B.: designed and analyzed all of the experiments, and critically revised the manuscript; A.H.: designed and analyzed all of the experiments and supervised the project, and critically revised the manuscript; A.S.: designed and analyzed all of the experiments and supervised the project, and wrote the manuscript; S.K., C.R.: helped with intraventricular infusion experiments and critically revised the manuscript; B.K.: helped with mRNA in situ hybridizations and critically revised the manuscript; J.M., M.F.: helped with histological analyses and critically revised the manuscript; L.W.: helped with histological analyses and wrote the manuscript; M.G.: helped with HPLC studies and critically revised the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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