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Regulation by commensal bacteria of neurogenesis in the subventricular zone of adult mouse brain

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Abstract

In the mouse olfactory bulb (OB), interneurons such as granule cells and periglomerular cells are continuously replaced by adult-born neurons, which are generated in the subventricular zone (SVZ) of the brain. We have now investigated the role of commensal bacteria in regulation of such neuronal cell turnover in the adult mouse brain. Administration of mixture of antibiotics to specific pathogen-free (SPF) mice markedly attenuated the incorporation of bromodeoxyuridine (BrdU) into the SVZ cells. The treatment with antibiotics also reduced newly generated BrdU-positive neurons in the mouse OB. In addition, the incorporation of BrdU into the SVZ cells of germ-free (GF) mice was markedly reduced compared to that apparent for SPF mice. In contrast, the reduced incorporation of BrdU into the SVZ cells of GF mice was recovered by their co-housing with SPF mice, suggesting that commensal bacteria promote the incorporation of BrdU into the SVZ cells. Finally, we found that administration of ampicillin markedly attenuated the incorporation of BrdU into the SVZ cells of SPF mice. Our results thus suggest that ampicillin-sensitive commensal bacteria regulate the neurogenesis in the SVZ of adult mouse brain.

Keywords: Adult neurogenesis, Subventricular zone, Olfactory bulb, Commensal bacteria, Antibiotics, Germ-free mouse

Abbreviations

ABPC, ampicillin;

Abx, antibiotic-treated SPF;

BrdU, bromodeoxyuridine;

co-GF, co-housed GF;

Ctrl, control SPF;

DAPI, 4', 6-diamidino-2-phenylindole;

DCX, doublecortin;

GCL, granule cell layer;

GF, germfree;

GFAP, glial fibrillary acidic protein;

IECs, intestinal epithelial cells;

IPL, internal plexiform layer;

LV, lateral ventricle;

mAb, monoclonal antibody;

Mash1, mammalian achaete-scute homolog 1;

MCL, mitral cell layer;

MNZ, metronidazole;

NeuN, neuronal nuclear antigen;

NM, neomycin;

OB, olfactory bulb;

SE, standard error;

SGZ, subgranular zone;

SPF, specific pathogen-free;

SVZ, subventricular zone;

VCM, vancomycin;

1. Introduction

Interneurons, such as granule cells and periglomerular cells, of the mammalian olfactory bulb (OB) are generated continuously from neural stem cells that reside in the subventricular zone (SVZ) of the lateral ventricle (LV) throughout adulthood [1]. A subpopulation of astrocytes, called type B1 cells, is thought to serve as the neural stem cells and to generate proliferating progeny, called type C cells, in the SVZ. Type C cells subsequently differentiate into neuroblasts, called type A cells [1]. Type A cells divide and migrate along the rostral migratory stream into the OB, where they differentiate into mature interneurons. The continuous production of new interneurons is thus balanced by the elimination of older interneurons, resulting in a rapid turnover of interneurons in the OB.

Such rapid turnover of interneurons in the OB is thought to be largely dependent on proliferation and migration of their progenitor cells in the SVZ. A previous study showed that the canonical Wnt pathway that stabilizes β-catenin promotes proliferation of both type B1 and type C cells in the SVZ [2]. Moreover, the non-canonical Wnt pathway (planar cell polarity pathway) is known to regulate the proliferation, migration and maturation of newly generated neurons [3-5]. In contrast, the Notch pathway is thought to be important for maintaining the type B1 cells [6]. In addition, receptors for epidermal growth factor and fibroblast growth factor are expressed in the SVZ, and ablation of their ligands reduces neurogenesis in the SVZ [7, 8], suggesting that these growth factors likely regulate neurogenesis in the SVZ.

Gut commensal bacteria have recently been demonstrated to play important roles in the homeostatic regulation of neuronal cells in the brain. Commensal bacteria are shown to regulate blood-brain barrier integrity, microglial maturation and myelination [9]. Furthermore, commensal bacteria are also shown to regulate the expression of neurotrophins, neurotransmitters and their receptors, and modulate behaviors [9]. In addition to the SVZ of the LV, adult neurogenesis occurs in the

subgranular zone (SGZ) of the hippocampal dentate gyrus [10]. Commensal bacteria are also suggested to regulate adult hippocampal neurogenesis [11]. Thus, we have now examined the role of commensal bacteria in regulation of neurogenesis at SVZ of the adult mouse brain.

2. Materials and methods

2.1. Mice

C57BL/6J and C57BL/6N male mice were obtained from CLEA Japan (Tokyo, Japan). These mice were maintained at the Institute for Experimental Animals at Kobe University Graduate School of Medicine under the specific pathogen-free (SPF) condition. Germ-free (GF) mice (C57BL/6N background) were obtained from CLEA Japan (Tokyo, Japan). For Reconstitution with normal commensal bacteria in GF mice, GF mice were co-housed with SPF mice from 4 to 9 week-old under the SPF condition. All animal experiments were performed according to Kobe University animal experimentation regulations.

2.2. Antibodies and reagents

A rat monoclonal antibody (mAb) to bromodeoxyuridine (BrdU) (#ab6326) was obtained from Abcam (Cambridge, MA). A mouse mAb to glial fibrillary acidic protein (GFAP) (#G3893) was obtained from Sigma-Aldrich (St. Louis, MO). A mouse mAb to mammalian achaete-scute homolog 1 (Mash1) (#556604) was obtained from BD Biosciences (San Diego, CA). A mouse mAb to doublecortin (#sc-271390) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A mouse mAb to NeuN (#MAB377) was obtained from Merck Millipore (Billerica, MA). Secondary antibodies labeled with Cy3 or Alexa488 for immunofluorescence analysis were obtained from Jackson ImmunoResearch (West Grove, PA) and ThermoFisher (Waltham, MA), respectively. Ampicillin, vancomycin, and metronidazole were obtained from Wako (Osaka, Japan). Neomycin sulfate and 4’,6-diamidino-2-phenylindole (DAPI) were obtained from Nacalai Tesque (Kyoto, Japan), and BrdU was from Sigma-Aldrich.

2.3. Antibiotic treatment

An antibiotic treatment to mice was performed as previously described [12,

13] but with a slight modification. In brief, mice were treated orally with an antibiotic cocktail (ampicillin, metronidazole, and neomycin each at 1 g/l, and vancomycin at 0.5 g/l) or with each antibiotic alone in drinking water from 4 to 9 week-old under the SPF condition.

2.4. BrdU incorporation

For analysis of the BrdU-incorporation into the SVZ, mice were injected intraperitoneally with BrdU (300 mg/kg). After 4, 8 or 24 h, brain samples for immunofluorescence analysis were prepared. For analysis of the number of BrdU-positive neurons in the OB, mice were injected intraperitoneally with BrdU (300 mg/kg), followed by provision of 1 mg/ml BrdU in drinking water for 14 days.

2.5. Immunofluorescence analysis

Preparation of the coronal sections of the SVZ was performed as previously described [14] but with a slight modification. In brief, 0–1400 μm rostral to the convergence of the anterior commissure was defined as containing the SVZ. Ten frozen sections with a thickness of 10 μm, each 150 μm apart (1:15 series), were prepared.

Coronal sections of the SVZ or the OB were subjected to immunofluorescence analysis with primary antibodies and fluorescent dye-labeled secondary antibodies with or without DAPI as described previously [15]. Labeled cells were counted under a fluorescence microscope (BX51; Olympus, Tokyo, Japan) or a laser scanning confocal microscope (LSM700; Carl Zeiss, Oberkochen, Germany), or analyzed with the use of ImageJ software (NIH).

For detection of BrdU, frozen sections were incubated for 30 min at 37°C with 1.5 N HCl, washed with 0.1 M borate buffer (pH 8.5), and then subjected to immunofluorescence analysis as described above.

2.6. Statistical analysis

Data are presented as means ± standard error (SE). Unpaired, two-tailed Student's t-tests were used to compare between 2 groups. One-way ANOVA followed by Tukey's tests were used to compare among 3 groups. Results were analyzed using GraphPad Prism software version 6.0 (GraphPad Software). A P value of <0.05 was considered statistically significant.

3. Results

3.1. Marked reduction of the BrdU-incorporation into the SVZ of antibiotic-treated SPF mice

To determine the effect of antibiotics in adult neurogenesis in the SVZ of the mouse brain, we first treated 4-week-old SPF mice with an antibiotic cocktail [ampicillin, vancomycin, metronidazole, and neomycin] in drinking water for 5 weeks. Such treatment was previously demonstrated to efficiently deplete commensal bacteria in the intestine [12, 16]. After antibiotic treatment, we examined the incorporation of BrdU into cells in the SVZ of mice. BrdU can be incorporated into DNA during DNA synthesis such as the S phase of the cell cycle and has been often used for labeling mitotic cells in studies of adult neurogenesis [17]. We found that BrdU-positive cells appeared at 4 h after a single injection of control SPF (Ctrl) mice with BrdU, and the number of BrdU-positive cells was increased over time (at 8 and 24 h) (**Fig. 1A and B**). In antibiotic-treated SPF (Abx) mice, we also detected BrdU-positive cells in the SVZ at 4-24 h after a single BrdU injection. However, the number of BrdU-positive cells in the SVZ was greatly reduced at 4 h and 8 h after a single BrdU injection in Abx mice compared with Ctrl mice (**Fig. 1A and B**). Thus, treatment of mice with antibiotics likely inhibits the proliferative activity of neuronal progenitor cells in the SVZ.

We next examined whether the antibiotic treatment affects the number of neuronal progenitor cells in the SVZ. The number of either glial fibrillary acidic protein (GFAP)-positive B1 cells (stem cells) (**Fig. 2A and B**), mammalian achaete-scute homolog 1 (Mash1)-positive C cells (transit-amplifying cells) (**Fig. 2C and D**) or doublecortin (DCX)-positive A cells (neuroblasts) (**Fig. 2E and F**) in the SVZ did not differ between Ctrl and Abx mice.

3.2. Marked reduction of the newly generated BrdU-positive neurons in the OB of antibiotic-treated SPF mice

Given that the treatment of mice with antibiotics reduced the number of BrdU-positive cells in the SVZ, we next examined the effect of antibiotic treatment on the number of newly generated neurons in the OB [1]. It was demonstrated that the number of BrdU-positive neurons in the mouse OB increases from 7 to 14 days after a BrdU injection [18]. To clarify the effect of antibiotics on the number of BrdU-incorporated neurons in the OB, either Ctrl or Abx mice were injected intraperitoneally with BrdU followed by administration of BrdU dissolved in drinking water for 14 days. Immunofluorescence staining for NeuN, a neuronal marker that labels interneurons but not mitral cells in the OB [19], showed no difference in the number of NeuN-positive cells in the area containing granule cell layer (GCL), internal plexiform layer (IPL) and mitral cell layer (MCL) of the OB between Ctrl and Abx mice (**Fig. 3A and B**). In contrast, the number of BrdU- and NeuN-double positive cells in the area containing GCL, IPL and MCL of OB was significantly reduced in Abx mice compared with Ctrl mice (**Fig. 3A and C**). These results thus suggest that antibiotic treatment reduces the newly generated BrdU-positive neurons in the mouse OB.

3.3. Marked reduction of the BrdU-incorporation into the SVZ of germ-free mice and ampicillin-treated SPF mice

To further clarify whether commensal bacteria regulate the BrdU-incorporation into the SVZ, we next examined the BrdU-incorporation into the SVZ of GF mice. The number of BrdU-positive cells at 4 h after a single BrdU injection was markedly reduced in the SVZ of GF mice compared with that of Ctrl mice (**Fig. 4A and B**). We next co-housed GF mice with Ctrl mice for 5 weeks and examined the incorporation of BrdU into their SVZ. Such co-housing of GF mice with Ctrl mice restored the number of BrdU-positive cells in the treated GF mice at 4 h after a single BrdU injection (**Fig. 4A and B**). Given that the co-housing is effective for transfer of commensal bacteria from SPF mice to GF mice [20], the result suggests that commensal

bacteria regulate the BrdU-incorporation into the SVZ of mice. Finally, we examined which of the antibiotics in the cocktail are responsible for suppression of the proliferative activity of neuronal progenitors in the SVZ. Treatment with ampicillin markedly reduced the incorporation of BrdU into the SVZ at 4 h after a single BrdU injection (**Fig. 4C**). In addition, treatment with vancomycin also reduced the incorporation of BrdU into the SVZ (**Fig. 4C**), such effect was not statistically significant, however. By contrast, treatment with either metronidazole or neomycin failed to affect the incorporation of BrdU into the SVZ (**Fig. 4D**). Thus, ampicillin-sensitive commensal bacteria likely promote the BrdU-incorporation into the SVZ of the adult mouse brain.

4. Discussion

We have here shown that the BrdU-incorporation into the SVZ was markedly reduced in Abx mice. We also found that the BrdU-incorporation into the SVZ was markedly attenuated in GF mice. By contrast, co-housing of GF mice with SPF mice, which is thought to transfer commensal bacteria from the latter mice to the former mice, restored the number of BrdU-positive cells in the SVZ of GF mice. Moreover, antibiotic treatment of mice reduced the newly generated BrdU-positive neurons in the OB. Thus, commensal bacteria likely promote the BrdU-incorporation, a marker for neurogenesis, into the neuronal progenitor cells in the SVZ. By contrast, antibiotic treatment did not significantly affect the numbers of either B1 stem cells, transit-amplifying C cells or neuroblasts (A cells) in the SVZ, suggesting that the effect of antibiotic treatment on neurogenesis is not enough to change the population of these neural progenitors.

The mechanism by which commensal bacteria regulate the BrdU-incorporation into the SVZ of adult mouse brain remains to be fully elucidated. We found that treatment with ampicillin or vancomycin reduced the BrdU-incorporation into the SVZ of mice. Given that these antibiotics are effective against Gram-positive

bacteria [21, 22], the result indicates that ampicillin-sensitive and Gram-positive bacteria likely promote the BrdU-incorporation into the SVZ of mice. We previously showed that Gram-positive commensal bacteria regulate the proliferative activity of intestinal epithelial cells (IECs) in intestinal crypts [13]. Moreover, such stimulatory effect of commensal bacteria is likely mediated by short-chain fatty acids, such as butyrate and acetate, as bacterial fermentation products. Indeed, subcutaneous injection of butyrate increases the BrdU-incorporation into the SVZ in cerebral ischemia induced rats [23]. Similar to the regulation of neuronal progenitor cells in the SVZ, Wnt, Notch and epidermal growth factor are thought to regulate progenitor of IECs in the crypt [24-27]. Thus, commensal bacteria likely regulate the neurogenesis in the SVZ of mouse brain through a similar mechanism for IECs in intestinal crypts. Further investigation is certainly necessary to clarify the molecular mechanisms by which commensal bacteria regulate the neurogenesis in the SVZ of adult mouse brain.

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Figure legends

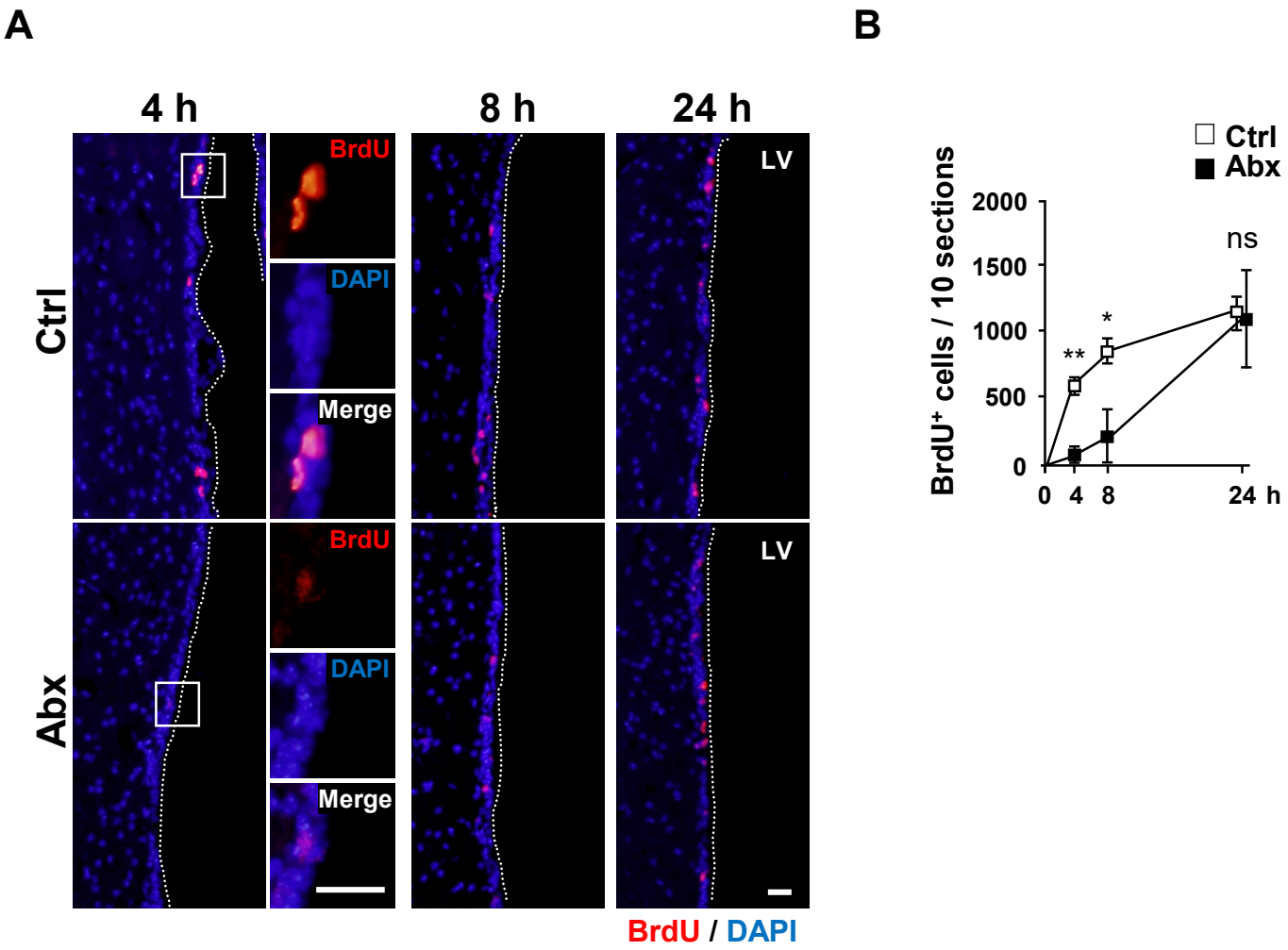
Fig. 1. Marked reduction of the BrdU-incorporation into the SVZ of antibiotic-treated SPF mice. (**A**) Coronal cryosections (10 μm) of the SVZ of control SPF (Ctrl) or antibiotic-treated SPF (Abx) mice were prepared at 4, 8, or 24 h after a single BrdU injection and were immunostained with an mAb to BrdU (red) and also stained with DAPI (blue). The boxed areas in the left panels for the sections prepared at 4 h after a single BrdU injection are shown at higher magnification in the right panels (upper, BrdU; middle, DAPI; lower, merge). Scale bars, 20 μm. (**B**) The number of BrdU-positive cells was determined from sections similar to those in (A). Dotted lines in (A) indicate boundaries of the SVZ and the LV. Ten sections were analyzed for each experiment (n = 3 mice, per group). Data are means ± SE. *, $P < 0.05$; **, $P < 0.01$; ns, not significant.

Fig. 2. The population of neuronal progenitor cells in the SVZ of antibiotic-treated SPF mice. (**A**, **C**, and **E**) Coronal cryosections (10 μm) of the SVZ of control SPF (Ctrl) or antibiotic-treated SPF (Abx) mice were immunostained with an mAb to GFAP (green A), Mash1 (green, C) or DCX (green, E) and also stained with DAPI (blue). Dotted lines indicate boundaries of the SVZ and the LV. The boxed areas in the panels are shown at higher magnification in the right panels (upper, GFAP (A), Mash1 (C), or DCX (E); middle, DAPI; lower, merge). Scale bars, 10 μm. (**B**, **D**, and **F**) The numbers of GFAP- (B), Mash1- (D), or DCX-positive cells (F) were determined from sections similar to those in (A), (C), or (E), respectively. Three areas were analyzed for each experiment (n = 3 mice, per group). Data are means ± SE. ns, not significant.

Fig. 3. Marked reduction of the newly generated BrdU-positive neurons in the OB of antibiotic-treated SPF mice. (**A**) Control SPF (Ctrl) or antibiotic-treated SPF (Abx) mice were injected intraperitoneally with BrdU followed by administration of BrdU

dissolved in drinking water for 14 days, after which coronal cryosections (5 μm) of the OB were immunostained with mAbs to BrdU (green) and to NeuN (red). The boxed areas in the upper panels are shown at higher magnification in the lower panels (left, BrdU; middle, NeuN; right, merge). Scale bars, 25 μm. Arrowheads indicate BrdU- and NeuN-positive cells. MCL, mitral cell layer; IPL, internal plexiform layer; GCL, granule cell layer. (**B**) The number of NeuN-positive cells per 10^4 μm^2 in the area containing GCL, IPL and MCL was determined from sections similar to those in (A). (**C**) The number of BrdU- and NeuN-positive cells per 10^4 μm^2 in the area containing GCL, IPL and MCL was determined from sections similar to those in (A). Nine areas were analyzed for each experiment (Ctrl, n = 6 mice; Abx, n = 5 mice). Data are means ± SE. *, $P < 0.05$; ns, not significant.

Fig. 4. Marked reduction of the BrdU-incorporation into the SVZ of germ-free mice and ampicillin-treated SPF mice. (**A**) Coronal cryosections of the SVZ of control SPF (Ctrl), germ-free (GF), and co-housed GF (co-GF) mice were prepared at 4 h after a single BrdU injection and were immunostained with an mAb to BrdU (red) and also stained with DAPI (blue). Dotted lines indicate boundaries of the SVZ and the LV. Scale bar, 20 μm. (**B**) The number of BrdU-positive cells was determined from sections similar to those in (A) (n = 4 per group). (**C**) The number of BrdU-positive cells in the SVZ of Ctrl, ampicillin (ABPC) treated-, or vancomycin (VCM) treated-mice at 4 h after a single BrdU injection (n = 4 per group). (**D**) The number of BrdU-positive cells in the SVZ of Ctrl, metronidazole (MNZ) treated-, or neomycin (NM) treated-mice at 4 h after a single BrdU injection (n = 3 per group). Ten sections with a thickness of 10 μm were analyzed for each experiment. Data are means ± SE. *, $P < 0.05$; ns, not significant.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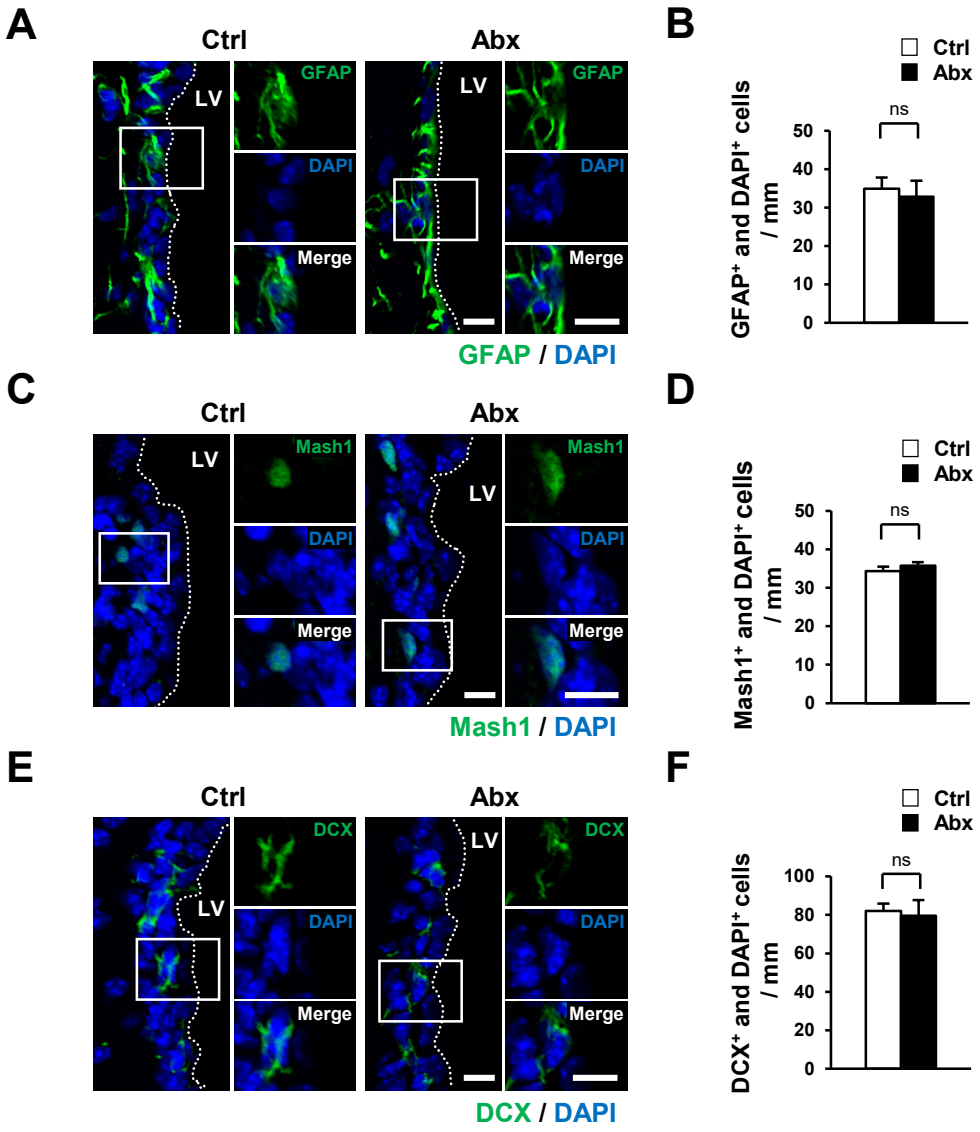
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Fig. 3
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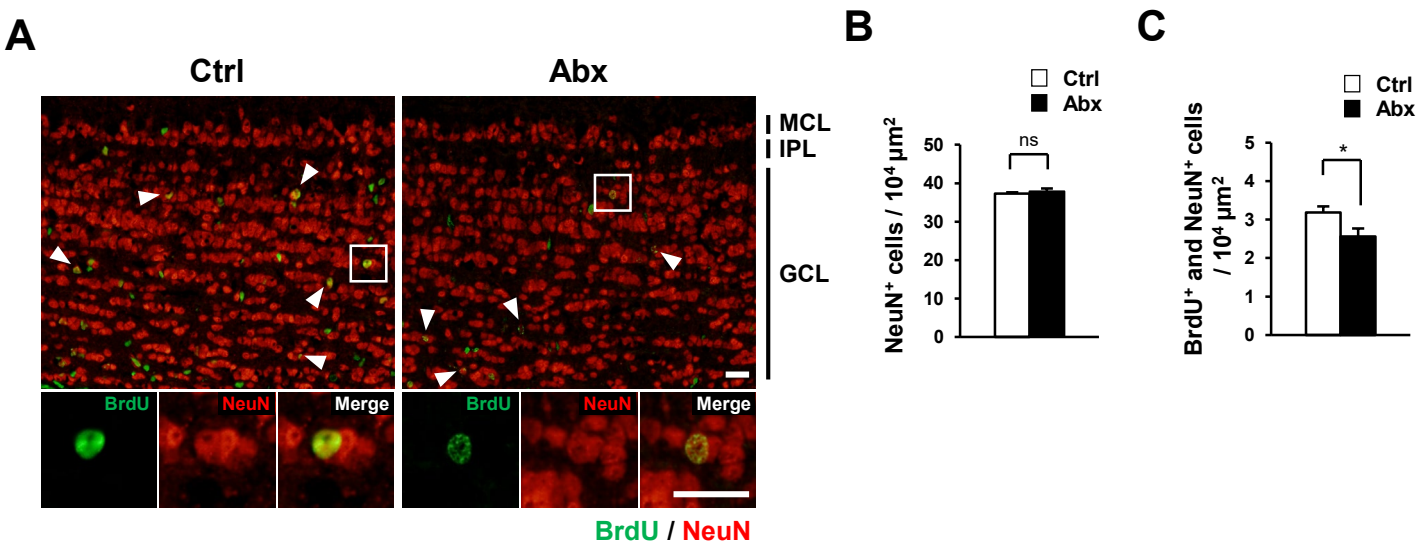

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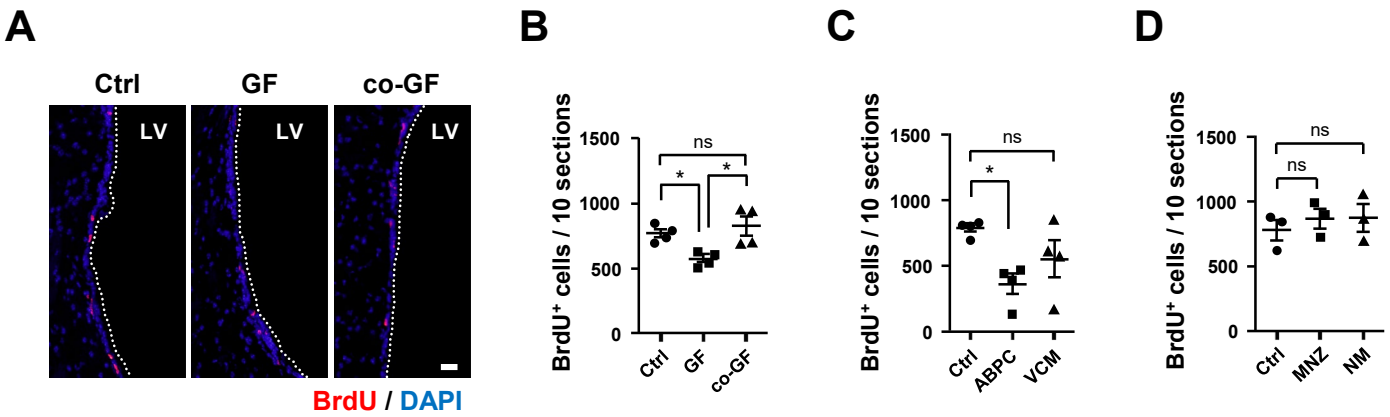

Conflict of interest

The authors declare that they have no conflicts of interests.