Population Dynamics of Neural Progenitor Cells during Aging in the Cerebral Cortex

Yuka Okada [a] and Koji Ohira [a]

[a] *Department of Food Science and Nutrition, Mukogawa Women's University, 6-46 Ikebiraki, Nishinomiya, Hyogo 663-8558, Japan*

*Corresponding author:

Koji Ohira

Department of Food Science and Nutrition, Mukogawa Women's University, 6-46 Ikebiraki, Nishinomiya, Hyogo 663-8558, Japan.

Email address: kohira@mukogawa-u.ac.jp

Abstract

Recent studies indicate that adult neurogenesis occurs in the cerebral cortex of rodents. Neural progenitor cells (NPCs) have been found in the adult cerebral cortex. These cells are expected to be regulated by various stimuli, including environmental enrichment, exercise, learning, and stress. However, it is unclear what stimuli can regulate cortical NPCs. In this study, we examined whether aging has an impact on population dynamics of NPCs in the murine cerebral cortex, using immunohistological staining for NPCs. The density of NPCs was kept from 5- to 12-month-old, dramatically decreased at 17-month-old, and thereafter maintained the same level until 24-month-old. Comparing the densities of NPCs in the cortical areas, such as the cingulate, primary motor, primary somatosensory, and insular cortices, we found that the degrees of decreased densities of NPCs in the cingulate and insular cortices were significantly smaller than those in the primary motor and somatosensory cortices. NPCs in aged cortex produced new neurons by ischemia. These results indicate that in aged mice, NPCs exist and produce new neurons in the cerebral cortex. Additionally, the extent of reduction of the density of NPCs in the cortices with higher cognitive functions may be less than that in the primary motor and somatosensory cortices.

Keywords

Adult neurogenesis; Aging; Cerebral cortex

1. Introduction

It has been established that new neurons are generated from neural progenitor cells (NPCs) and stem cells (NSCs) and contribute to neural plasticity throughout life [1]. NSCs can produce NPCs and glial progenitor cells, and NPCs and glial progenitor cells provide new differentiated neurons and central glial cells, such as astrocytes and oligodendrocytes, respectively. Currently, NPCs and NSCs are found in some regions of the adult mammalian brains, such as the hippocampal dentate gyrus, subventricular zone, and cerebral cortex [1,2].

In the subventricular zone and the dentate gyrus, NSCs and NPCs show self-renewal and continue to produce new neurons even under healthy conditions [3,4]. Besides, newborn neurons in the olfactory bulb and the hippocampus are needed for olfactory memory [5,6], and contextual and spatial memory [7–9], respectively. These findings suggest that new neurons play an important role in the structural and functional plasticity of the adult brain.

The cerebral cortex is involved in several functions of the body and mind, including determination, intelligence, personality, planning, and motor control [10–12]. Interestingly, several studies suggest that adult neurogenesis occurs in the cerebral cortex of adult mammals [11–18]. Although cortical adult neurogenesis under healthy conditions remains controversial [19], new neurons have been reported to be induced by pathological stimuli, such as ischemia and lesion [12,14,16–18]. In addition, NPCs, which can produce new neurons in the cerebral cortex, have been first identified in the layer 1 of the cerebral cortex,

and can generate inhibitory interneurons by ischemic stimuli. The cells are designated as layer 1 inhibitory neuron progenitor cells (L1-INP cells) [2,18]. Recently, chronic treatments with antidepressants have been reported to generate new neurons from L1-INP cells [20]. However, it is not clear whether other factors can induce adult neurogenesis of L1-INP cells.

Hippocampal adult neurogenesis is governed by various stimuli, including learning, exercise, environmental enrichment, and stress [21]. Among them, aging has been reported to be associated with a substantial decline of adult neurogenesis in hippocampus [22]. In this study, we examined whether the density of L1-INP cells are decreased during aging in the cerebral cortex, using immunostaining. We also investigated the capability of L1-INP cells for neuron production in the aged cortex.

2. Materials and methods

2.1 Animals

Male C57BL/6J mice at 5, 12, 17, and 24 months of age (Charles River Laboratories Japan, Yokohama, Japan; 4 mice each age) were utilized. For infection with retrovirus vectors, we used male C57BL/6J mice at 5 and 24 months of age (n = 16, 20, respectively). Mice were housed (4 mice per cage) in a room with a 12 hours light/dark cycle (lights on at 8:00 a.m.) with access to food and water ad libitum. All of the animal experiments were

approved by the Animal Care and Use Committee of Mukogawa Women's University, based on Mukogawa Women's University's guidelines for the ethical treatment of laboratory animal (FSN-03-2015-01-A), and conducted in accordance with the principles of the Declaration of Helsinki.

2.2. Retrovirus-mediated GFP labeling of newly generated neurons

The retrovirus vector construct, pDON-5 Esyn-GFP-WPRE, was used in this study [18]. The retrovirus vectors were produced according to the manufacturer's instructions accompanying the retrovirus packaging kit (Takara Bio, Otsu, Japan). The resulting viral particles in the culture supernatant were adjusted to 1.0×10^6 transducing units/ml.

The virus injection was performed as described previously [18]. Briefly, the virus solution (0.2 ml per site) was stereotaxically injected by air pressure through a glass micropipette attached to Picospritzer III (Parker, Cleveland, OH) into the primary motor cortex (0.8-1.8 mm anterior to bregma, 1-2 mm lateral, and 0.2 mm depth below the cortical surface) [23]. One hemisphere received a total of 5 μl of the virus solution (25 injection sites in 1.0 mm × 1.0 mm square).

2.3. Ischemia treatment

Ischemia was induced according to the previous reports [18,24]. Briefly, 2 days after the virus injection, both common carotid arteries (CCAs) were transiently occluded with

clamps for 10 min. Control animals were treated identically, except for occlusion of CCAs. The mice were allowed to survive for 4 weeks after ischemia.

2.4. Immunostaining

Fixation and immunofluorescence staining were performed as previously described [20]. Briefly, mice were deeply anesthetized with chloral hydrate (245 mg/kg, intraperitoneally) and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). The brain samples were mounted in Tissue-Tek (Miles, Elkhart, IN), frozen, and cut coronally into 50-μm-thick coronal sections, using a sliding microtome (model LS-113; Yamato-Kouki Co.; Saitama, Japan). After washing in PBS for 1 h, sections were incubated at 4°C for 48 h or at room temperature overnight with the indicated primary antibodies.

For fluorescent staining, after washing in PBS for 1 h, the sections were incubated at room temperature for 1 h with secondary antibodies conjugated with fluorescent dyes. After washing in PBS, sections were mounted on glass slides coated with 3-aminopropyltriethoxysilane and embedded with Permafluor (Thermo Scientific, Pittsburgh, PA). Confocal laser-scanning microscopy (A1+, Nikon, Tokyo, Japan) was used to obtain images of the stained sections.

For immunostaining with ABC method, the sections were incubated with a biotinylated secondary antibody at room temperature for 24 h. The immunoreactive sites were visualized with the avidine-biotin complex peroxidase method using the avidin-biotin

complex kit (ABC kit: Vector Laboratories). A 3,3'-diamino benzidine tetrahydrochloride, 4HC1 solution containing 0.3% nickel ammonium sulfate in 0.05 M Tris-HCl, pH 7.6, was used as the substrate for peroxidase.

2.5. Antibodies

The following primary antibodies were used: mouse monoclonal antibody for Ki67 (Antibody Registry ID, AB393778; 1:20, BD Biosciences, San Jose, CA); rabbit polyclonal antibodies for GABA (Antibody Registry ID, AB477652; 1:1000, Sigma-Aldrich, St. Louis, MO) and GFP (Antibody Registry ID, AB10073917; 1:1000, Thermo Fisher Scientific). The following secondary antibodies were used: goat anti-mouse IgG Alexa Fluor 594 (1:200, Life Technologies, Carlsbad, CA), goat anti-rabbit IgG Alexa Fluor 488 (1:200, Life Technologies), goat biotinylated anti-rabbit IgG (1:200, Vector Laboratories).

2.6. Image analysis

Positive cell counting was carried out in the images obtained. Area sizes of cortical layer 1 in all captured images were measured with ImageJ. For the quantification of the numbers of immunopositive cells, we used ImageJ with WCIF ImageJ bundle (http://www.uhnres.utoronto.ca/facilities/wcif/). There are many GABA+ structures in the tissue images, such as GABA+ cells and GABA+ neurites. GABA+ neurites were excluded in cell counting. In addition, tissue images were converted into 8-bit black and white

images. Image thresholds were automatically determined by a plugin “maximum entropy threshold”, and the binary images were obtained. Once the images were segmented, GABA+ cells were visualized. Round or orbital cell bodies were designated as GABA+ cells, while thin-long structures were designated as neurites. GABA+ cells were first determined, and Ki67+/GABA+ cells were identified. Densities of L1-INP cells are given as cell numbers/mm^3 (4 mice at each time point; means ± S.E.M).

To produce a distribution map of L1-INP cells, all labeled L1-INP cells, which are contained in 6 serial sections (total 300 μm), were piled up to a single plane of brain atlas (see Fig. 2A).

For quantification of new neurons, which were stained by 3,3’-diamino benzidine tetrahydrochloride, 4HCl (DAB) and nickel ammonium sulfate, all GFP-immunostained new neurons were counted in all serial sections of the virus vector-infected regions.

2.7. Statistical analysis

Changes in densities of L1-INP cells during aging of the cerebral cortex were investigated using one-way ANOVA. Differences between cortical areas and time points were compared using two-way ANOVA. Decreased density of L1-INP cells in the cingulate and insular cortices and the primary motor and somatosensory cortices was compared by *t* test. Significant main effects and/or interactions were followed by Tukey's post hoc analyses to examine group or time differences. Adult neurogenesis of L1-INP cells

in interaction of two factors, aging and ischemia, was compared by two-way ANOVA. Values are given as means ± S.E.M. GraphPad Prism (version 6, GraphPad Software, La Jolla, CA) was used to analyze all data. For all statistical analyses used, the alpha level was set at $P < 0.05$.

3. Results

3.1. Reduction in the density of L1-INP cells during aging

To examine whether the density of L1-INP cells decreased during aging in the cerebral cortex, we used cerebral cortex at ages of 5, 12, 17, and 24 months. GABA and Ki67, molecular markers for L1-INP cells, double-positive (GABA+/Ki67+) cells in the cortical layer 1 were designated as L1-INP cells [18,20]. We found that GABA+/Ki67+ round cells existed in the layer 1 (Fig. 1), which is in agreement with previous reports [18,20]. In addition, the cells were identified in all time points of ages that we tested. Morphological changes seemed not to be induced during aging. Representative images of L1-INP cells were shown in Fig. 1B-D and Supplementary Fig. 1.

We quantified the density of L1-INP cells at each time point. The density of L1-INP cells was kept by 12 months of age, and was drastically reduced at 17 months of age ($P<0.0001$; Fig. 1E). There was not any significant change of density of L1-INP cells between 17 and 24 months of ages.

3.2. Distributional changes of L1-INP cells in cortical areas during aging

Next, we examined if there were any differences in densities of L1-INP cells during aging between cortical areas, cingulate, primary motor, primary somatosensory, and insular cortices. Six 50-μm-thick sections were superimposed to create the images (Fig. 2A). Densities of L1-INP cells declined dramatically between 12 and 17 months of ages. However, labeled L1-INP cells seemed to be uniformly distributed across the layer 1 of cerebral cortex during aging.

To compare the densities of L1-INP cells during aging between cortical areas, we calculated the densities of L1-INP cells in each area and time point. As shown in Fig. 2B, there were significant changes in distributions of L1-INP cells during aging among areas. At 5 months of age, densities of L1-INP cells in the somatosensory cortices were higher than those in the cingulate and insular cortices (somatosensory to cingulate, $P<0.05$; somatosensory to insular, $P<0.01$; Fig. 2B). In contrast, at 24 months of age, we hardly found differences between densities of L1-INP cells in the cingulate and insular cortices and those in the primary motor and somatosensory cortices. We further compared the declines of the densities of L1-INP cells between the primary cortex (the primary motor plus somatosensory cortices) and higher order cortex (the cingulate plus insular cortices) between 12 and 24 months of ages. As expected, the degree of reduced number of L1-INP cells in primary cortex was much smaller than that in higher order cortex ($t(6) = 3.082$,

P<0.05; Fig. 3). There were no significant differences in the degrees of reduced numbers of L1-INP cells between the cingulate cortex and the insular cortex (cingulate cortex, 10.13 ± 5.90 vs. insular cortex, 9.98 ± 2.55; t(6) = 0.0237, P=0.98), nor between the primary motor cortex and the primary somatosensory cortex (motor, 22.3 ± 1.37; somatosensory, 22.5 ± 4.35; t(6) = 0.0439, P=0.97).

3.3. Generation of new neurons from L1-INP cells in aged cortex

We examined whether L1-INP cells in aged cortex produced new neurons. L1-INP cells in the motor cortex of 5 month-old and 24 month-old mice were labeled with GFP, which was delivered by recombinant retrovirus vectors [18,20]. Recombinant retrovirus vectors express membrane-targeted GFP under human synapsin I promoter, a well-known neuron-specific promoter. Only proliferating cells incorporate the transgene into their genome via retrovirus vectors, suggesting that L1-INP cells were infected with retrovirus vectors when the vectors were injected to the cortical layer 1 [18,20,25]. At 2 days after injections of virus vectors, mice were treated for 10 min with global ischemia, which makes L1-INP cells generate new neurons [18]. In the cortex of 5 month-old mice, the density of GFP+ new neurons was remarkably increased to 14-folds by ischemic stimuli (P<0.0001; Fig. 4). In the cortex of 24 month-old mice, low levels of adult neurogenesis were found in control mice. Interestingly, ischemia invoked a significant increased production of new neurons (P<0.0001), which is larger than a number of new neurons in 5 month-control mice

($P<0.01$).

4. Discussion

L1-INP cells have been reported to be found in young and middle-aged rodents [18,20]. In this study, we found that L1-INP cells existed in the cerebral cortex of aged mice. Densities of L1-INP cells were retained by 12 months of age, drastically declined at 17 months of age, and after that kept until 24 months of age. In addition, the extent of the decrease in density of L1-INP cells in the cingulate and insular cortices was significantly smaller than that in the primary motor and somatosensory cortices. In aged cortex, L1-INP cells had the ability to generate new neurons in responding to ischemia stimuli.

In the hippocampus, adult neurogenesis persists throughout adulthood, but the rate of generation of new neurons dramatically decreases as the animal becomes older [26]. A 40-fold reduction was found when comparing 2- to 10-month-old mice [27]. As corresponding to that, the age-related decline in NPC proliferation in mice begins at 1-2 months of age and progressively reduces thereafter [27–29]. Namely, in the hippocampus, the decrease in adult neurogenesis seems to be almost hyperbolic, with a very sharp decline early in life and a rather low but persistent level for the remainder of the life span [28,30]. As shown in Fig. 1, the density of L1-INP cells is 11.4 cells/mm^3 at 5 months of age. We previously found that there were approximately 15 cells/mm^3 of L1-INP cells at 2 months after birth

[20], which is similar to the density of L1-INP cells at 5 months of age. These findings indicate that the density of L1-INP cells might show no significant changes from 2 to 12 months of age. Thereafter, the drastic decrease in L1-INP cells occurred between 12 and 17 months of ages, and the density of L1-INP cells was maintained until 24 months of age. Thus, age-related decline in the density of L1-INP cells may be delayed, compared to hippocampal NSCs/NPCs.

In this study, we found that the degree of reduced number of L1-INP cells in the cingulate and insular cortices was significantly smaller than that in the primary motor and somatosensory cortices between 12 and 24 months of age (Fig. 2). The primary motor cortex is dedicated to transmitting impulses to the voluntary muscles, while the primary somatosensory cortex receives the somatic sensory input via the thalamus [10]. These regions have somewhat simpler functions, termed the primary cortices [10]. The cingulate cortex having connections with the hippocampus and amygdala is a part of the orbitomedial prefrontal cortex, which is involved in limbic system and functions in impulse control and personality, and appears to be most associated with mood (particularly depression and mania) [31]. The insular cortex has broad functions, such as perception, motor control, self-awareness, and auditory processing of language [32–34]. The cingulate and insular cortices are involved in higher-order brain functions. L1-INP cells tend to be left in higher-order cortical areas during aging, compared to the primary cortex. The animals that we used throughout this study were kept in normal cages with cage mates during aging. This means

that functions of the primary cortex may not have to change according to the invariable environment of cages. In the hippocampus, physical exercise can up-regulate adult neurogenesis. Thus, densities of L1-INP cells in the primary cortex, especially in the primary motor cortex, would be maintained during aging. On the other hand, the animals, which were kept with cage mates, were constantly exposed across the lifespan to affective states that arose when certain animal interacted with others and that depended on the social context. The cingulate and insular cortices are involved in such social emotions [31,35]. In fact, social poor condition, such as isolation stress, has negative effect on adult neurogenesis of hippocampus [36,37], which have strong connections with cingulate and insular cortices. Thus, these suggest that brain plasticity of the cingulate and insular cortices may be maintained at higher levels than that of the primary cortex. The degrees of decreased numbers of L1-INP cells in the cortices may reflect the requirement of brain plasticity in each cortical area.

New neurons were generated from L1-INP cells in aged cortex, even under normal conditions, albeit low levels of neurogenesis. Ischemia treatments significantly up-regulated cortical adult neurogenesis in aged cortex. These results show that endogenous NPCs that can be activated by brain insults are consistently maintained during aging, suggesting that these NPCs might be useful for endogenous regenerative therapy for brain damage. In future studies, the mechanism for proliferation and differentiation of L1-INP cells will be needed to be clarified. If the mechanism will be clear, treatments and/or drugs

that can control the proliferation and differentiation of L1-INP cells might be developed based on the mechanism.

Abbreviations

L1-INP cells, layer 1 inhibitory neuron progenitor cells; NPCs, neural progenitor cells; NSCs, neural stem cells

Conflict of interest

The authors declare that they have no conflicts of interests.

Acknowledgements

This work was supported by JSPS KAKENHI [grant numbers JP26430044, JP17K07084].

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Figure Legends

Fig. 1. L1-INP cells in the aged cerebral cortex. (A) Schematic representation of the cerebral cortex. A boxed area indicates the area of (B). The drawing was quoted from the mouse brain atlas [23]. (B) GABA-immunostained cells in the primary motor cortex at 5 months of age. The higher magnification of the boxed area in (B) appears in (C). (C) A representative image of L1-INP cells. (D) A Z-plane of serial optical planes of L1-INP cell. L1-INP cells, which is marked by arrowheads, are stained with anti-GABA (green) and anti-Ki67 (red) antibodies (C, D). (E) Quantification of the numbers of L1-INP cells during aging in the cerebral cortex. The data are shown as means ± S.E.M. ****, P<0.0001. Values are analyzed by one-way ANOVA and Tukey's *post hoc* test, n=4 each.

Fig 2. Distributional changes of L1-INP cells in the cortical regions of cerebral cortex during aging. (A) Distributions of L1-INP cells in the cerebral cortex at 5, 12, 17, and 24 months of age. Each circle means a L1-INP cell, which are co-labelled by anti-Ki67 and anti-GABA. Labeled L1-INP cells in six 50-μm-thick sections are superimposed to create the schemas. The drawings were quoted from the mouse brain atlas [23]. (B) Quantification of the densities of L1-INP cells during aging in four cortical areas. The data are shown as means ± S.E.M. Values are analyzed by two-way ANOVA and Tukey's *post hoc* test, n=4 each. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001.

Fig. 3. Differences in the degrees of decreased densities of L1-INP cells between higher order cortex (the cingulate plus insular cortices) and primary cortex (the primary motor plus somatosensory cortices). (A) Quantification of the densities of L1-INP cells during aging in higher order cortex and primary cortex. The data are averages from the cingulate plus insular cortices (black) or the primary motor plus somatosensory cortices (gray). Values are analyzed by two-way ANOVA and Tukey's *post hoc* test, n=4 each. **, $P<0.01$; ***, $P<0.001$. ctx, cortex. (B) Decreased density of L1-INP cells in higher order cortex is lower than that in primary cortex. The data (means ± S.E.M.) means decreased densities of L1-INP cells from 12 to 24 months of age. Values are analyzed by Student's t-test, n=4 each. *, $P<0.05$.

Fig. 4. Generation of new neurons from L1-INP cells in 5 month-old and 24 month-old mice. (A) Images of new neurons in the cerebral cortex treated with ischemia. Low levels of neurogenesis are shown in the control mice at both ages, whereas many new neurons are observed in ischemia-treated mice at both ages. New neurons are stained with anti-GFP (white and black arrowheads). A higher magnification of new neuron is displayed in an inset by a black arrowhead. (B) Quantification of cortical adult neurogenesis at 5 and 24 month-old. In the cortex at both ages, adult neurogenesis was up-regulated by ischemia treatments. The data are shown as means ± S.E.M. Values are analyzed by two-way

ANOVA and Tukey's *post hoc* test (5 month-old, 8 control and 8 ischemia-treated mice; 24 month-old, 9 control and 11 ischemia-treated mice). **, $P<0.01$; ****, $P<0.0001$.

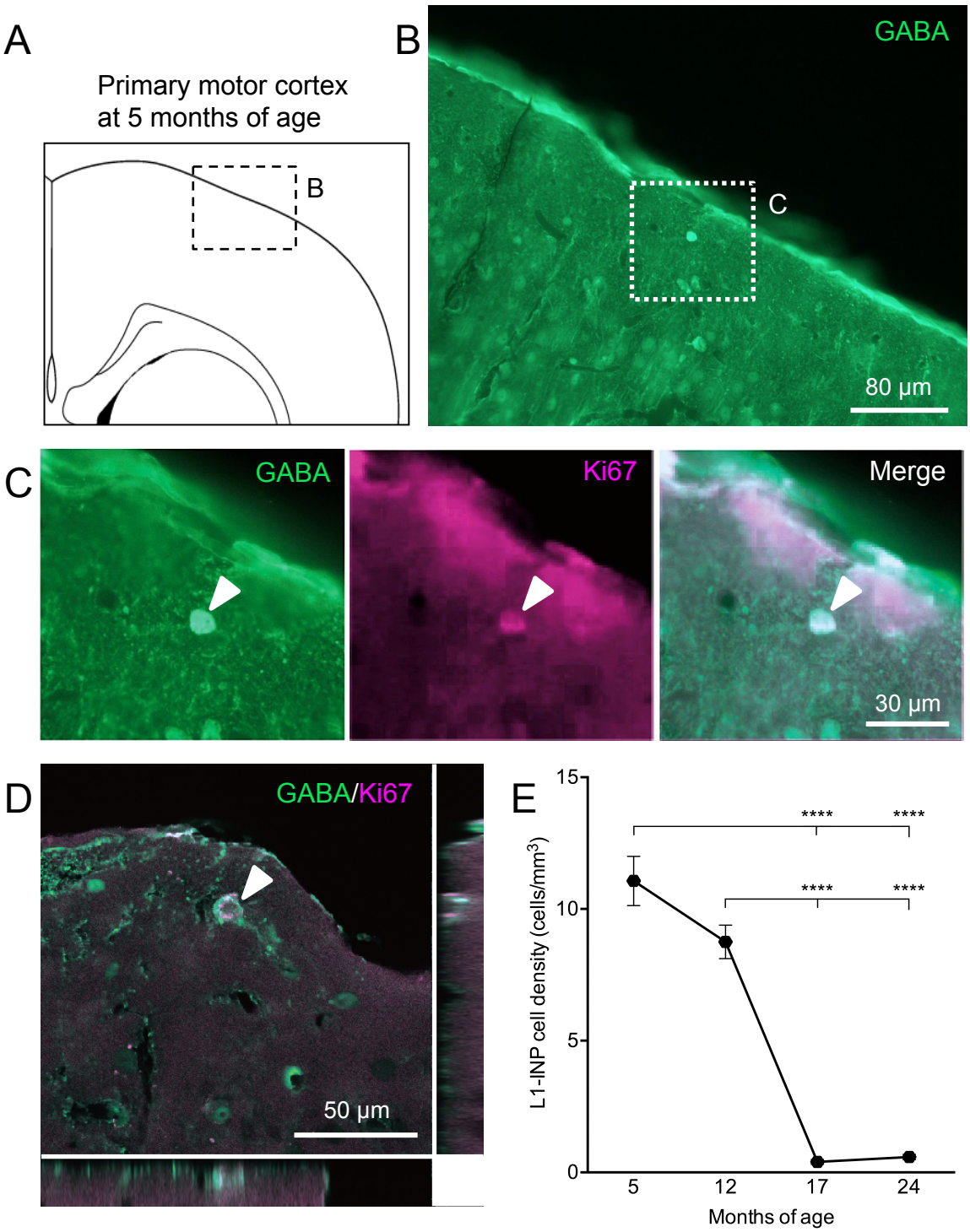

A
Primary motor cortex
at 5 months of age
B
B
GABA
C
80 μm
C
GABA
Ki67
Merge
30 μm
D
GABA/Ki67
50 μm
E
L1-INP cell density (cells/mm3)
15
10
5
0
****
****
****
****
5
12
17
24
Months of age

Fig. 1

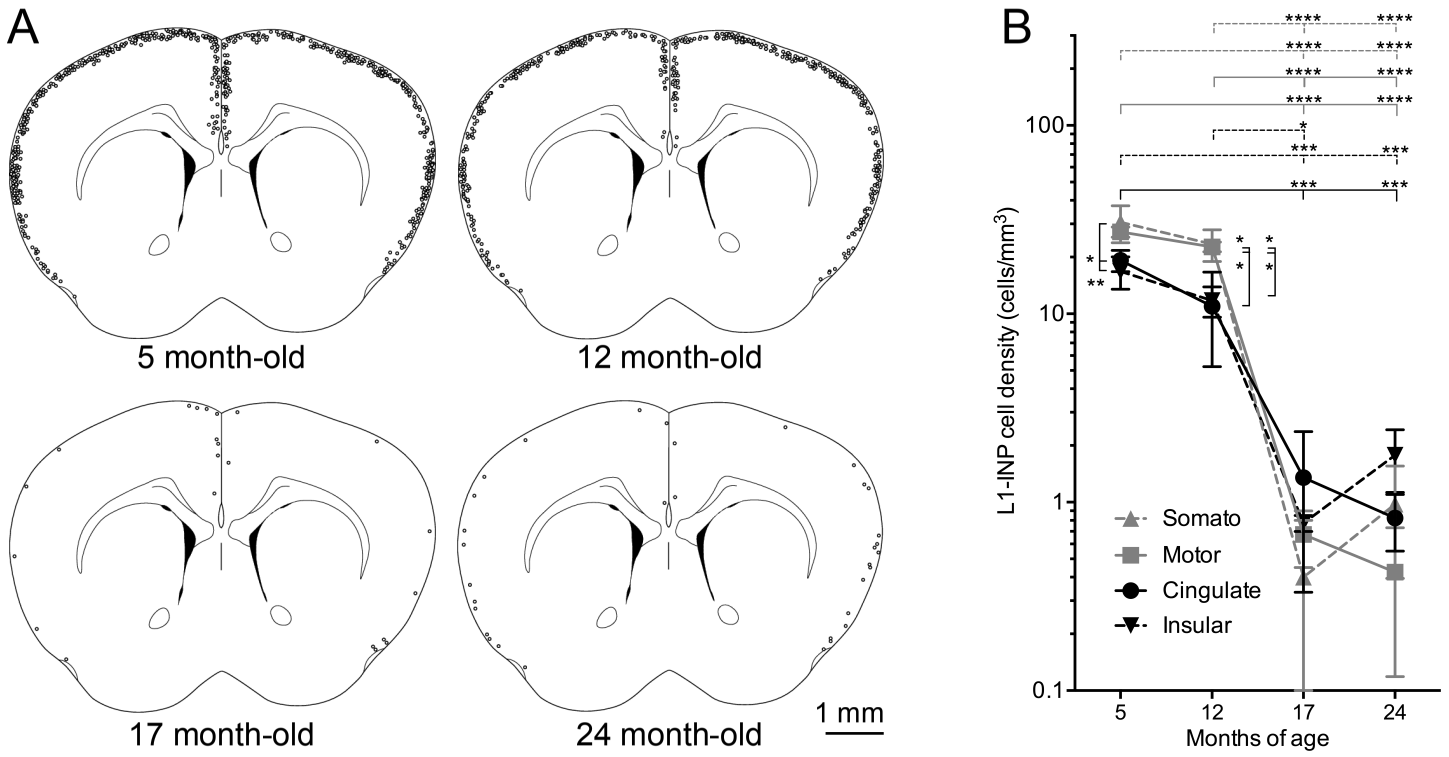

A
5 month-old
12 month-old
17 month-old
24 month-old
1 mm
B
L1-INP cell density (cells/mm3)
100
10
1
0.1
5
12
17
24
Months of age
Somato
Motor
Cingulate
Insular

Fig. 2

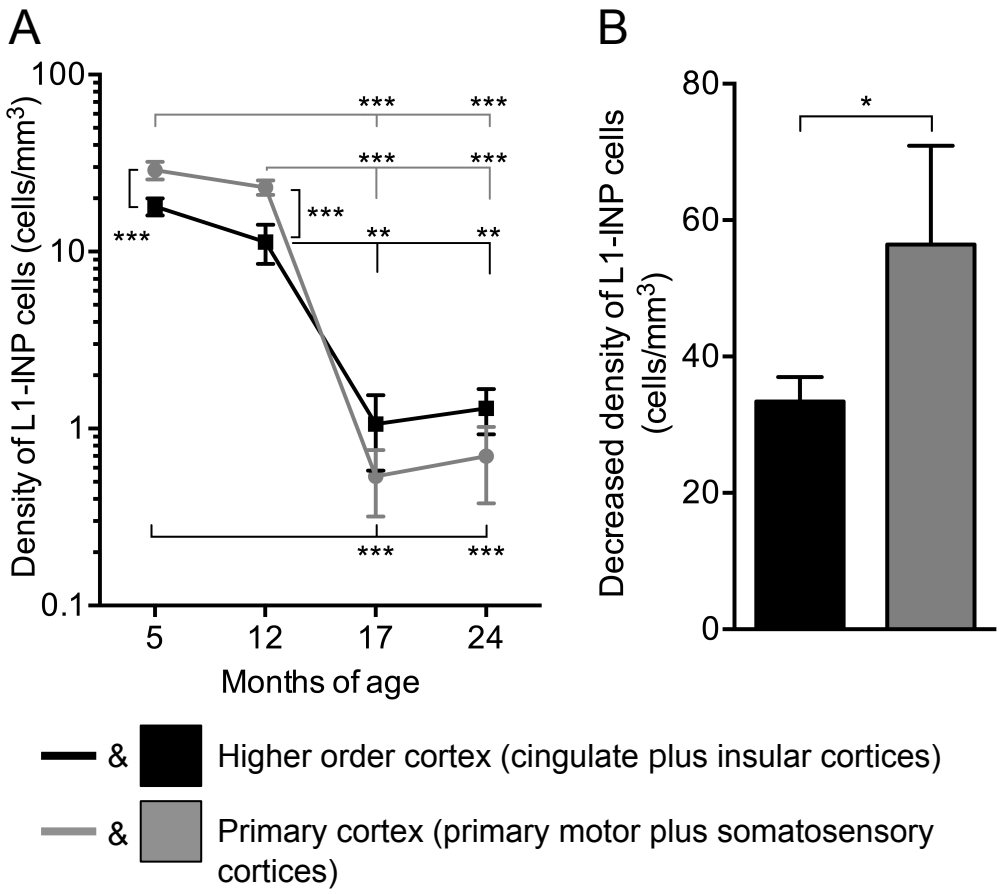

A
B
100
10
1
0.1
Density of L1-INP cells (cells/mm3)
5
12
17
24
Months of age
***
**
80
60
40
20
0
Decreased density of L1-INP cells (cells/mm3)
*
& Higher order cortex (cingulate plus insular cortices)
& Primary cortex (primary motor plus somatosensory cortices)

Fig. 3

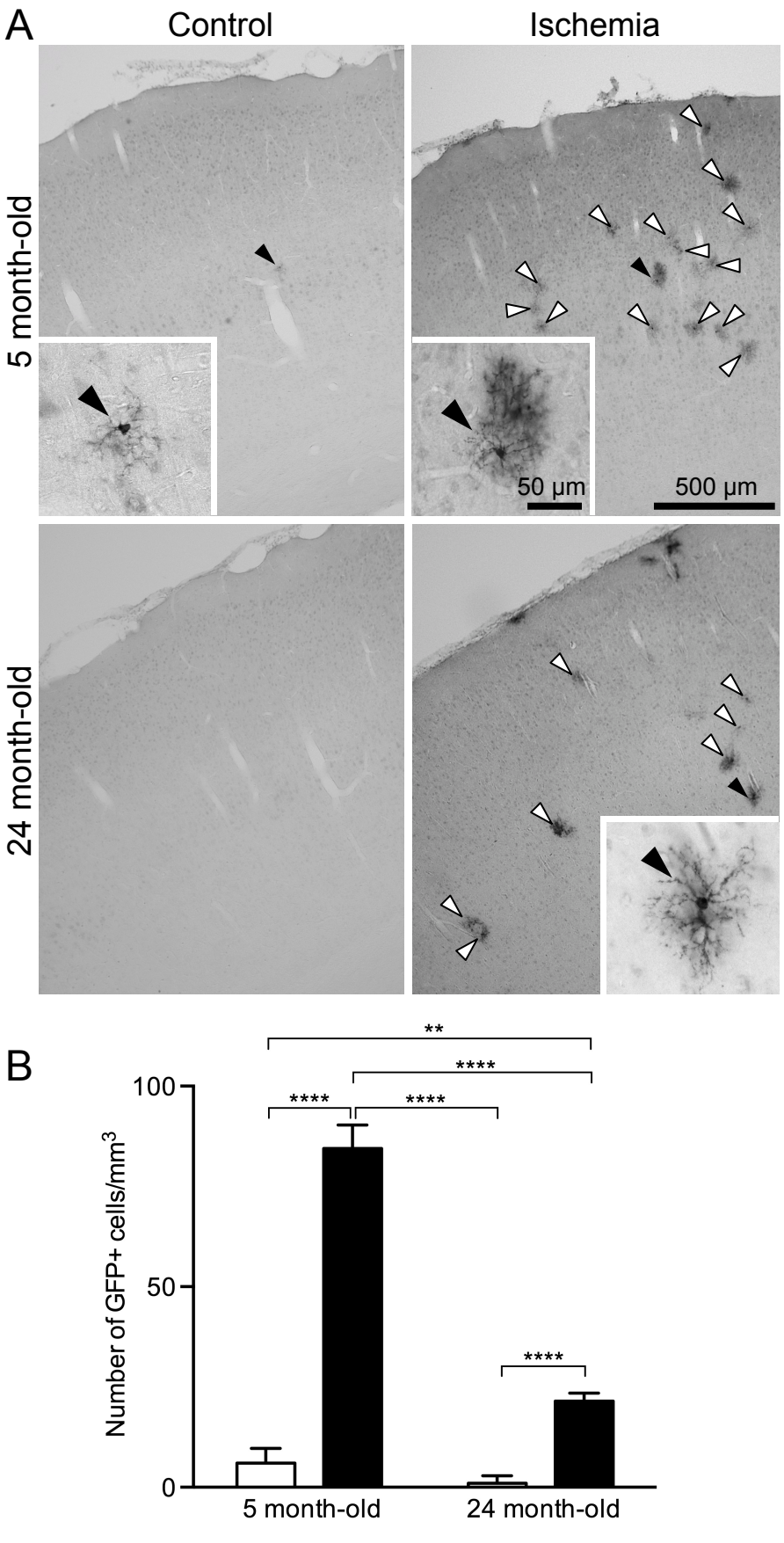
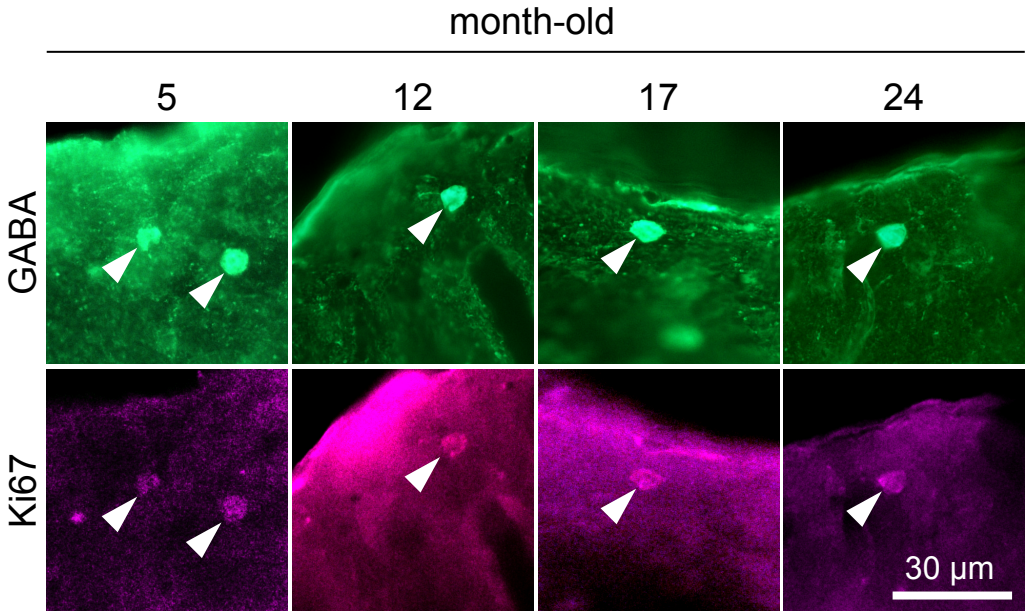

A
Control
Ischemia
5 month-old
24 month-old
50 µm
500 µm
B
Number of GFP+ cells/mm3
100
50
0
**
****
****
****
****
5 month-old
24 month-old

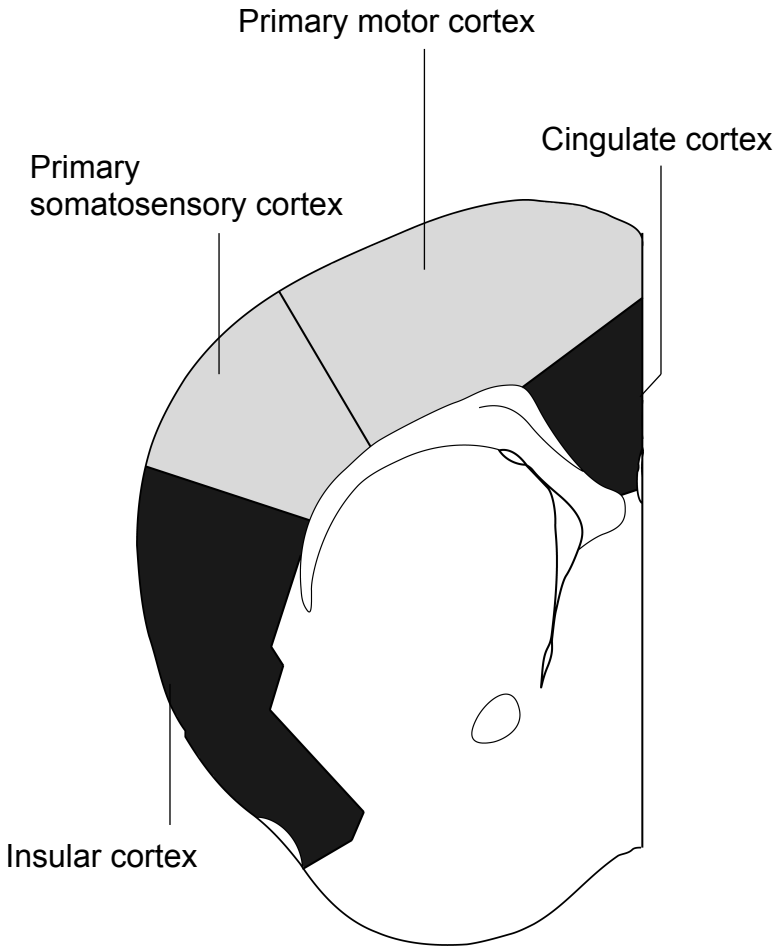
Fig. 4

Supplementary Fig. 1



Supplementary Fig. 1. Representative images of L1-INP cells during aging of the cerebral cortex. Arrowheads indicate L1-INP cells, which are labelled with anti-GABA (green) and anti-Ki67 (magenta).

Supplementary Fig. 2



Supplementary Fig. 2. Schematic representation of cortical areas, the primary somatosensory cortex, primary motor cortex, cingulate cortex, and insular cortex. ctx, cortex. The drawing was quoted from the mouse brain atlas [23].