Title Page

Title:
c-jun is differentially expressed in embryonic and adult neural precursor cells

Authors:
Fumiaki Kawashima[1], Kengo Saito[1], Hirofumi Kurata[1, 2], Yoshihiro Maegaki[2], Tetsuji Mori[1*]

1) Department of Biological Regulation, School of Health Science, Faculty of Medicine, Tottori University, 86 Nishi-cho, Yonago, Tottori 683-8503, Japan
2) Division of Child Neurology, Institute of Neurological Sciences, Faculty of Medicine, Tottori University, 86 Nishi-cho, Yonago, Tottori 683-8503, Japan

***Corresponding Author:**
Tetsuji Mori
Department of Biological Regulation, School of Health Science, Faculty of Medicine, Tottori University, 86 Nishi-cho, Tottori 683-8503, Japan
Tel & Fax: +81-589-38-6352
e-mail: mori-te@med.tottori-u.ac.jp

Acknowledgement:
We thank Professor Dr. Masayoshi Okada for his critical review. This work was supported by JSPS KAKENHI Grant-in-Aid for Scientific Research(C), Grant Number: 25430044.

Abstract

c-jun, a major component of AP-1 transcription factor, has a wide variety of functions. In the embryonic brain, c-jun mRNA is abundantly expressed in germinal layers around the ventricles. Although the subventricular zone (SVZ) of the adult brain is a derivative of embryonic germinal layers and contains neural precursor cells (NPCs), the c-jun expression pattern is not clear. To study the function of c-jun in adult neurogenesis, we analyzed c-jun expression in the adult SVZ by immunohistochemistry and compared it with that of the embryonic brain. We found that almost all proliferating embryonic NPCs expressed c-jun, but the number of c-jun immunopositive cells among proliferating adult NPCs was about half. In addition, c-jun was hardly expressed in post-mitotic migrating neurons in the embryonic brain, but the majority of c-jun immunopositive cells were tangentially migrating neuroblasts heading toward the olfactory bulb in the adult brain. In addition, status epilepticus is known to enhance the transient proliferation of adult NPCs, but the c-jun expression pattern was not significantly affected. These expression patterns suggest that c-jun has a pivotal role in the proliferation of embryonic NPCs, but it has also other roles in adult neurogenesis.

Keywords

c-jun, immunohistochemistry, development, adult neurogenesis, status epilepticus

Introduction

Activator protein-1 (AP-1) is a transcription factor consisting of homo- or heterodimers composed of proteins from various families such as Jun (c-jun, JunB, JunD) and fos (c-fos, FosB, Fra1, Fra2) (Herdegen and Waetzig 2001). Among them, c-jun is a key component of the AP-1 transcription factor and can form heterodimers with other transcription factors (Raivich and Behrens 2006). As a member of immediate early genes, c-jun mRNA and protein are immediately upregulated in response to various stimuli (Raivich and Behrens 2006). c-jun plays important roles in a wide variety of phenomena, including cell cycle control, apoptosis, and axogenesis (Wilkinson et al. 1989; Herdegen and Waetzig 2001).

During development, c-jun mRNA is widely expressed in rodent embryonic tissues. In the central nervous system (CNS), the level of c-jun mRNA is high (Wilkinson et al. 1989). In particular, its mRNA is abundantly detected in germinal layers including the ventricular and subventricular zone, consisting of actively proliferating neural precursor cells (NPCs) of the telencephalon at embryonic day 14.5 (Wilkinson et al. 1989). c-jun mRNA expression level is maximal at postnatal 15 (P15) but gradually declines toward the adult stage (Mellström et al. 1991). From the expression pattern analysis, it has been suggested that c-jun might be involved mainly in the proliferation of NPCs in the embryonic brain.

In the adult brain, basal c-jun mRNA and protein expression is detected in a small fraction of mature cells (Herdegen and Leah 1998). The adult brain also contains proliferating NPCs. There are commonly accepted two neurogenic regions: one is the subventricular zone (SVZ), lining the lateral ventricle, and the another is the subgranular zone of the hippocampus. The NPCs in the SVZ sequentially differentiate from slowly cycling neural stem cells (Type B cells), transit amplifying cells (Type C cells), and neuroblasts (Type A cells). Subsequently, Type A cells tangentially migrate with limited cycles of proliferation to the olfactory bulb (OB) through the rostral migratory stream (RMS). Then they migrate radially and finally differentiate into granular neurons or periglomerular neurons in the OB (Mori et al. 2005; Lledo et al. 2006; Ponti et al. 2013).

In the embryonic brain, radial glia serve as apical progenitors and produce basal progenitors, and they constitute the ventricular zone and subventricular zone, respectively (Wilsch-Bräuninger et al. 2016). Type B cells in the adult SVZ derive from radial glia, and they share astrocyte properties including molecular marker expression (Merkle et al. 2004; Mori et al. 2005). Because almost all cells in the germinal layers of the embryonic telencephalon seem to express c-jun from in situ hybridization data, there

is a possibility that neural precursors in the adult SVZ also express c-jun. But a detailed c-jun expression pattern in the adult SVZ has not been examined.

Adult neurogenesis is tightly regulated. However, proliferation and migration can be disrupted under some pathological conditions, such as stroke, traumatic injury, and seizures (Parent et al. 2002; Zhang et al. 2004; Mori et al. 2012). In the status epilepticus (SE) model rodents, proliferation of NPCs in the adult SVZ is transiently enhanced (Parent et al. 2002). Although a lot of studies have shown that c-jun expression is induced in neurons and glial cells immediately after seizures in the adult brain (Herdegen and Leah 1998), it has not been examined whether c-jun expression is upregulated in the adult neurogenic regions after seizures.

In the present study, we aimed to speculate the function of c-jun in adult neurogenesis. 1) We examined the expression pattern of c-jun in the adult SVZ by immunohistochemistry. And because of the similarity between embryonic and adult NPCs as described above, we compared the c-jun expression pattern in these NPCs. 2) In addition, we examined whether c-jun expression could be changed in the SE model mice. Our findings indicated that c-jun protein was continually expressed in the germinal zones throughout life, but a differential expression pattern was found in the embryonic and adult NPCs. Moreover, c-jun expression was tightly regulated in the adult NPCs after induction of SE.

Materials and Methods

Animals

Timed pregnant ICR mice were used for analysis of the embryonic brain. The day when a vaginal plug was detected was defined as embryonic day 0 (E0). Six- or seven-week-old male ICR mice were used for analysis of the adult brain. Mice were supplied by Japan SLC. Inc. (Hamamatsu, Japan). Mice were maintained under 12-h bright/right cycles. All experiments were performed in compliance with the Guidelines for Animal Experimentation, Faculty of Medicine, Tottori University under the International Guiding Principles for Biomedical Research Involving Animals.

Status epilepticus model mice

Pilocarpine-induced SE model mice were created as follows. Scopolamine methyl bromide (scopolamine; Sigma, St. Louis, MO, USA), pilocarpine hydrochloride (pilocarpine; Nacalai tesque, Kyoto, Japan), and phenobarbital (Sigma) were dissolved

in phosphate-buffered saline (PBS) at 0.2 mg/ml, 50 mg/ml, and 10 mg/ml, respectively. All drugs were administrated intraperitoneally. Scopolamine was administrated at a dose of 1 mg/kg to prevent peripheral responses to pilocarpine. Pilocarpine was administrated at 300 mg/kg 30 min after the scopolamine. An identical volume of scopolamine and PBS was administered to control mice. The behavior of mice was observed for 30 min after pilocarpine administration. Most mice started to exhibit myoclonus within 10 min. Seizures developed into a continuous tremor and finally into generalized clonic seizures with falling that lasted for several seconds. Subsequently, the mice exhibited continuous wet-dog shakes and periodical generalized clonic seizures. Mice that exhibited a milder behavioral change (no response, sniffing, head-nodding, myoclonus or a single attack of generalized clonic seizures without continuous wet-dog shakes) were not used for the analysis. To stop the seizures, phenobarbital was administrated at a dose of 50 mg/kg 1 h after pilocarpine administration. Mice were fixed at 1, 8, or 24 h after pilocarpine administration. For the 1-h mouse group, phenobarbital was not administrated.

BrdU labeling experiment

To detect actively proliferating cells, BrdU labelling was administered. BrdU (Tokyo Kasei, Tokyo, Japan) was dissolved in PBS at 10 mg/ml and injected intraperitoneally at a dose of 50 mg/mg. Mice were analyzed 2 h after BrdU injection.

Histological procedures

Tissure preparation

Timed pregnant female mice were deeply anesthetized with 4% chloral hydrate (600 mg/kg), and embryonic brains were immersion-fixed with 4% formaldehyde for 4 h. Adult mice were deeply anesthetized with 4% chloral hydrate (600 mg/kg) and perfused transcardially with PBS, followed by 4% formaldehyde in PBS. The brains were removed, post-fixed with the same fixative overnight. All brains were cryoprotected with 20% sucrose in PBS, embedded in Super Cryo Mount (Muto Pure Chemicals, Tokyo, Japan), snap frozen on dry ice, and cut transversely using a cryostat. Coronal sections were made as 12 μm-thick sections attached to the glass slides for embryonic brain analysis or 30 μm-thick free floating sections for adult brain analysis. The sections were then processed for immunohistochemistry.

Antibodies and immunohistochemistry

The following primary antibodies were used: rabbit monoclonal anti-c-jun (clone

60A8, 1:2000; Cell Signaling Technology, Danvers, MA, USA)(Mazzitelli et al. 2011), mouse anti-GFAP (1:400; Sigma), mouse anti-Mash1 (1:2000; BD Biosciences, San Jose, CA, USA), guinea pig anti-DCX (1:2000; Millipore, Temecula, CA, USA), goat anti-MCM2 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-NeuN (1:200, Millipore), mouse anti-Glutamine Synthase (GS, 1:500, BD Biosciences), mouse anti-S100β (1:1000, Sigma), mouse anti-GST-π (1:2000, BD Biosciences), goat anti-IbaI (1:400, Abcam, Cambridge, UK), rat anti-BrdU (1:200, Abcam).

In some experiments, special treatments were needed. To detect astrocytes, a cocktail of anti-GS and anti-S100β antibodies (markers for astrocyte) was used because astrocytes are highly heterogeneous, and a single marker cannot label all astrocytes (Kimelberg 2004). To retrieve the antigenicity of MCM2, sections were incubated in L.A.B. solution (Polysciences, Warrington, PA, USA) for 5 min at room temperature. To detect BrdU labeled cells, sections were pretreated with 2 M HCl for 30 min at room temperature followed by neutralization with 0.1 M sodium borate (pH 8.5) and three washes with PBS.

Primary antibodies were detected using species-specific donkey secondary antibodies conjugated to Alexa- Fluor 488 (1:400; Invitrogen, Carlsbad, CA, USA), Alexa-Fluor 555 (1:400, Invitrogen), or Cy2 (1:200; Jackson ImmunoResearch, West Grove, PA, USA). To visualize nuclei, stained sections were mounted onto glass slides using a medium containing 100mM DTT, 50% glycerol, and 5 μg/ml Hoechst 33258. In some experiments, primary antibodies were detected with a chromogenic reaction. Sections were incubated in 0.3% H_2O_2 for 5 min and incubated with each primary antibody, followed by incubation with secondary antibodies conjugated to biotin (1:200, Vector Laboratories, Burlingame, CA, USA). The chromogenic reaction was performed with an ABC kit (Vector laboratories) and 0.05% DAB solution with 0.05% Nickel chloride and 0.015% H_2O_2 for 5 min.

Image acquisition

Images were acquired using an epi-fluorescent microscope (TE2000, Nikon, Tokyo, Japan) equipped with a digital camera (Wraycam-SR130M, Wraymer Microscope, Osaka, Japan) or with a light microscope (Opti-photo, Nikon) equipped with a digital camera (EOS kiss X8i, Cannon, Japan). Single optical confocal microscopy images were acquired using an LSM 780 microscope with a 40x objective lens (Carl Zeiss, Oberkochen, Germany).

Quantification analysis

For quantification analysis of the embryonic brain, confocal microscopy images were acquired. Every tenth section was collected, and three sections from each embryo were used for quantification. For quantification of the adult brain, every tenth coronal section was collected from 1.18 mm to -0.10 mm anteroposterior to the bregma, and three sections were analyzed from each mouse. For quantification of BrdU+ cells in the anterior SVZ of the SE mice, randomly selected three sections from 1.42 mm to 1.72 mm anteroposterior to the bregma were analyzed from each mouse.

The number of immunopositive cells with a nuclear-staining pattern, such as c-jun, MCM2 and BrdU, was quantified with ImageJ using the particle analysis function (https://imagej.nih.gov/ij/). The area was also calculated with ImageJ. DCX+ cells were counted manually. The densities of c-jun+ cells are presented as the mean ± standard deviation (SD). Level of significance was determined using the two-tailed student's *t*-test. Three mice were analyzed in each group. Statistical significance was set at $p < 0.05$. The statistical analysis was performed using R, a free statistical software package (http://www.R-project.org).

Results

c-jun protein was expressed in the germinal regions in the brain throughout life

In the past, almost all c-jun expression pattern analysis in the embryonic brain was done by in situ hybridization, and the c-jun protein expression pattern was not clear. Our immunohistochemistry results mostly supported those of mRNA expression, but there were also inconsistent results. In the telencephalon, c-jun immunopositive (+) cells were localized in the germinal layers, close to the ventricle at E14 (Figs. 1a–d). This region corresponds to the ventricular and subventricular zones, containing actively proliferating NPCs. Interestingly, the c-jun expression level was higher in the dorsal and ventral regions than in the middle regions (Figs. 1a–d), and this regional difference was not detected in later embryonic stages. This differential expression in the dorsoventral axis had not been described in in situ hybridization experiments (Wilkinson et al. 1989). Next, to confirm c-jun protein expression in the proliferating cells, we performed double immunostaining with anti-c-jun and anti-MCM2, a marker of G1 phase of the cell cycle (Maslov et al. 2004). Most of the c-jun+ cells were MCM2+, indicating that c-jun+ cells were proliferating cells (Figs. 1e–g, arrows). A notable point was that there were only a few c-jun single+ cells in the cortical mantle layers at E14 (Fig.1h, arrows),

where a scattered expression of c-jun mRNA was reported (Wilkinson et al. 1989). Afterward, the number of c-jun+ cells in the cortical mantle layers increased in the later embryonic and postnatal stages (Figs.1i and 1j), supporting the former in situ hybridization studies (Wilkinson et al. 1989; Mellström et al. 1991). In the postnatal stages, the c-jun protein expression pattern did not largely change in the germinal layers (Fig. 1j).

We found that c-jun protein expression continued into the adult stage in the SVZ (Fig. 2a). In contrast to the embryonic brain, c-jun immunoreactivity occurred even in the dorsoventral axis of the SVZ (Fig. 2a). Double immunostaining analysis revealed that there were considerable numbers of c-jun and MCM2 co-expressing cells (Figs. 2b–d, arrows). To compare the quantification analysis of c-jun+ cells in the germinal zones in the embryonic and adult brain, we analyzed the dorsolateral region of the lateral ventricle: a border between the dorsal and ventral pallium. We took advantage of selecting this region, because the morphology of the lateral ventricle dramatically changes during development, but this dorsolateral region principally conserves its morphology during development. Because we found that some ependymal cells, which comprised a single layer lining the adult lateral ventricle and were not proliferating (Spassky et al. 2005), were also c-jun+, we excluded these c-jun+ ependymal cells from the quantification analysis (Figs. 2b–d, dashed line). Quantification analysis revealed that the ratio of MCM2+ cells among c-jun+ cells was not significantly different between the embryonic germinal layers and the adult SVZ: about 70% of c-jun+ cells were proliferating in both stages (Fig. 2e). On the other hand, the ratio of c-jun+ cells among MCM2+ cells was significantly lower in the adult SVZ than in the embryonic germinal layers (Fig. 2f). These differential expression patterns between embryonic and adult NPCs suggest that c-jun is not be essential in proliferation of adult NPCs and may have other function(s).

The majority of c-jun+ cells were DCX+ in the adult SVZ

Next, we examined the possibility that c-jun might be involved in cell migration in adult NPCs, because c-jun can enhance cell migration in some types of cells (Jiao et al. 2010; Lu et al. 2010). First, we analyzed whether c-jun could be expressed in post-mitotic migrating cells in the embryonic brain, where there were a few c-jun+ cells in the mantle layers as described above (Fig. 1h). The mantle layers in the embryonic cortex contain radially and tangentially post-mitotic migrating cells both from the dorsal and ventral germinal layers to form the cerebral cortex (Luhmann et al. 2015). And these migrating cells express DCX (Gleeson et al. 1999). At E14, DCX

immunoreactivity was detected outside the germinal layers (Figs 3a–c), but few c-jun/DCX double+ cells were detected (Figs. 1h and 3a–c). On the other hand, a small number of c-jun/DCX double+ cells were scattered in the germinal layers (2.12±1.10% among c-jun+ cells, n = 3), especially those close to the surface of the ventricle (Online Resource 1, arrows). Because DCX is also involved in the proper orientation of mitotic spindle angle in embryonic NPCs (Pramparo et al. 2010), these c-jun/DCX double+ cells in the germinal layers should not be post-mitotic migrating cells.

NPCs in the adult SVZ are defined by molecular marker expression; Type B cells (GFAP+), Type C cells (Mash1+), and Type A cells (DCX+) (Ponti et al. 2013). To identify c-jun+ cells, we examined marker expression in c-jun+ cells in the adult SVZ. Virtually no immunoreactivity was detected in GFAP+ cells (Figs. 3d–f). Although c-jun was expressed both in Mash1+ (Figs. 3g–i) and DCX+ cells (Figs. 3j–l), the majority of c-jun+ cells (61.86±10.49% among c-jun+ cells, n = 3) were DCX+. Thus, there is a possibility that c-jun could have some role in cell migration in adult neurogenesis.

c-jun was expressed in tangentially but not radially migrating adult NPCs

Next, we analyzed the c-jun expression pattern along the rostrocaudal axis of the SVZ-RMS-OB system, the migration pathway of the NPCs. The density of c-jun+ cells gradually decreased in the OB, but there was a substantial number of c-jun+ cells in the core of the OB (Figs. 4a–c). In the granule cell layer (GCL) of the OB, there were also many intensely labeled c-jun+ cells (Fig. 4c), but they did not express DCX (Figs. 4d–f, open arrowheads). c-jun+ cells in the GCL were NeuN+ mature neurons (Figs. 4g–I, arrows).

Type A cells migrate with several rounds of mitosis (Ponti et al. 2013). Thus, we examined several markers for cell proliferation in the SVZ-RMS-OB system. MCM2 was expressed in the entire SVZ-RMS and even in the core of the OB (Figs. 5a–c). On the other hand, BrdU+ cells were detected mainly in the SVZ (Figs. 5d–f). Our BrdU labelling protocol, with single injection and short-term survival period, effectively labels actively proliferating cells (Codega et al. 2014). The lengths of S phase and M phase of the cell cycle are relatively stable, but the length of G1 phase is variable: slowly cycling cells have a longer G1 phase (Takahashi et al. 1995; Zhang et al. 2008). In addition, both slowly and fast cycling cells can be detected with anti-MCM2 antibody (Maslov et al. 2004). These results indicate that the proliferation activity of Type A cells gradually decreased and almost completely ceased after the cells exited the RMS. Our results indicated that c-jun was expressed in tangentially migrating and

slowly cycling cells in the RMS. In addition, it is still difficult to determine whether c-jun is involved in cell migration in the adult neurogenesis.

The density of c-jun expression in the SVZ did not change after pilocarpine-induced SE

To further examine the role of c-jun in adult neurogenesis, we analyzed pilocarpine-induced SE mice for whether c-jun protein expression could be upregulated in the SVZ, correlating with the transiently enhanced proliferation of NPCs (Online Resource 3) (Parent et al. 2002).

In the control brain, a large number of c-jun+ cells were detected in the SVZ (Figs. 6a and 6b). However, in other regions, the number of c-jun+ cells was at a basal level (Fig. 6a). In the SE brain, c-jun immunoreactivity was also detected in the SVZ (Fig. 6c and 6d), and prominent upregulation was detected in neurons and glial cells all over the brain (Figs. 6d, arrows, and 6e–h). However, the density of c-jun+ cells in the SVZ did not significantly change (Fig. 6i).

Discussion

In the present study, to speculate the function of c-jun in adult neurogenesis, we examined the c-jun expression pattern in the adult SVZ by immunohistochemistry. In addition, the c-jun expression pattern was compared between the embryonic and adult brain.

Embryonic brain

During mid-gestation periods, NPCs actively proliferate in the germinal layers, and after their final division, post-mitotic young neurons radially or tangentially migrate to their final destinations (Luhmann et al. 2015). In this process, c-jun protein was mainly detected in the proliferating cells in the ventricular and subventricular zone of the telencephalon. This immunohistochemical data support the former in situ hybridization data. Indeed, c-jun is expressed in the various types of proliferating cells, including tumors (Ouafik et al. 2009; Blau et al. 2012). These results suggest that in the embryonic brain, c-jun might have important roles in cell cycle regulation in actively proliferating cells.

On the other hand, post-mitotic migrating young neurons were not c-jun+, but some neurons after finishing migration expressed c-jun again. Because basal and

stimuli-induced c-jun protein expression can be detected in mature neurons in the adult brain (Herdegen and Leah 1998), there should be another function of c-jun in post mitotic neurons. Dendrites and spines are not static and are newly formed or retracted depending on brain activity (Trachtenberg et al. 2002; Lee et al. 2006). In fact, c-jun can induce neurite extension (Herdegen and Waetzig 2001; Raivich et al. 2004). Furthermore, the c-jun protein expression level reaches its peak around P15 (Herdegen and Leah 1998). In this period, neuronal connections or synaptic formation in the cortex is abundantly formed, then gradually selected, or pruned (Pinto et al. 2013; Miyamoto et al. 2016). Thus, basal c-jun expression in neurons might reflect neuronal maturation or activity.

There was a discrepancy between the present results and former reports. It was reported that a scattering of c-jun mRNA was detected in the mantle layers at E14 (Wilkinson et al. 1989); however, we could detect only a few c-jun+ cells in the mantle layers. The gap between the transcription and translation of c-jun is relative short because it is a member of the immediate early genes (Herdegen and Leah 1998). Therefore, the gap may be much longer in the developing brain due to posttranscriptional regulation (Bolognani and Perrone-Bizzozero 2008).

Adult brain

Through the postnatal stages, c-jun protein expression remained in the germinal layers around the lateral ventricle. However, its expression pattern in the adult brain was distinct from that of the embryonic brain. First, about 70% of c-jun+ cells were MCM2+ in both stages, but the ratio of c-jun+ cells among MCM2+ cells was significantly lower in the adult SVZ than in the embryonic germinal layers. Second, c-jun was expressed in a sub-population of Type C cells (fast proliferating cells) in the SVZ and in slowly cycling Type A cells in the RMS. These results suggest that c-jun might not only promote the fast proliferation of NPCs but also be involved in other functions in the adult brain.

Our analysis revealed that, in the adult brain, the majority of c-jun+ cells were the tangentially migrating Type A cells in the SVZ. We found that radially migrating DCX+ cells in the OB did not express c-jun. However, a sub-population of mature granular neurons did express c-jun again after they had differentiated into mature neurons. Thus, c-jun might have some roles in cell migration. In fact, it was reported that deletion of endogenous c-jun caused a reduction in migratory velocity and invasiveness in mammary tumor epithelial cells (Jiao et al. 2010). Moreover, GDNF induces the cell migration of glioma via the JNK/c-jun pathway (Lu et al. 2010).

Alternatively, c-jun might have some roles in directing the tangential migration of NPCs. Tangential and radial migration of adult NPCs is regulated by distinct factors (Lledo and Saghatelyan 2005; Lalli 2014). Among them, a direction switch is induced by down-regulation of the sphingosine 1-phosphate receptor 1 (S1P1), resulting in downregulation of NCAM, and β1 integrin expression, in migrating neuroblasts (Alfonso et al. 2015). The c-jun expression pattern in the RMS along the rostrocaudal axis suggests that c-jun might be responsible for tangential but not radial migration in the SVZ-OB system.

SE brain

c-jun protein upregulation has been thoroughly analyzed by immunohistochemistry in the adult brain after various stimulations (Herdegen and Leah 1998), but it remains unclear whether c-jun expression could be changed in the adult SVZ by some stimuli. Supporting former studies, c-jun protein was upregulated both in neurons and glial cells in other parts of the "non-neurogenic" brain regions, such as the striatum, after induction of SE. However, we found that c-jun expression was stable at least 24 h after induction of SE. These results clearly suggest that c-jun expression is under the control of a different mechanism in neurogenic and non-neurogenic regions, and c-jun expression is tightly regulated in the SVZ.

There have been many reports indicating that proliferation of NPCs in the SVZ can be affected by excess neuronal excitation under pathological conditions (Zhang et al. 2004; Zhang et al. 2008; Kernie and Parent 2010). Pilocarpine-induced SE leads to transient upregulation of NPC proliferation (Parent et al. 2002). On the other hand, although migration of NPCs toward the OB is partially dispersed in the SE brain, the gross migration direction and the migration speed in the SE brain is normal (Parent et al. 2002). Considering the present study indicating the stable c-jun expression after induction of SE, it is also suggested that c-jun might not only be involved in cell cycle regulation in adult neurogenesis.

Function of c-jun in adult neurogenesis

Our results suggest that c-jun might have distinct functions in embryonic and adult neurogenesis. In the adult SVZ-RMS-OB system, c-jun could be involved in the migration of NPCs, in addition to proliferation. c-jun has a wide variety of functions, and its function in adult neurogenesis is complicated. It is important to note that c-jun was expressed in a sub-population of adult NPCs. Adult NPCs in the SVZ are highly heterogeneous with regional identity and diverse differentiation fates (Merkle et al.

2007), and c-jun could have different functions in each small sub-population of adult NPCs. Unfortunately, there not exist any specific molecular markers to distinguish each sub-population.

During adult neurogenesis, nearly half of newly generated cells undergo cell death (Biebl et al. 2000; Petreanu and Alvarez-Buylla 2002). Because c-jun induces apoptosis through the JNK/c-jun pathway (Herdegen and Waetzig 2001) and because phosphorylated JNK, an active form of JNK, is also expressed in the SVZ (data not shown), there is a possibility that c-jun might have some roles in the elimination of newly generated cells in the SVZ and RMS. However, because the number of TUNEL+ apoptotic cell in the SVZ and RMS is much lower than the number of c-jun+ cells (Biebl et al. 2000), induction of cell death by c-jun could be limited if possible.

To examine the function of c-jun in vivo, knockout mice of c-jun should be useful. Brain specific c-jun conditional knockout (cKO) mice were generated (Raivich et al. 2004). They have a normal gross brain morphology (Raivich et al. 2004) and can survive because c-jun function is compensated for by other AP-1 transcription factor(s) at least in the developing and normal adult brain (Passegué et al. 2002). But those cKO mice have impairment of axonal regeneration at the adult stage (Raivich et al. 2004). There are still possibilities that those cKO mice have some defectiveness in the adult NPCs. Further detailed analysis is required to elucidate the function of c-jun in adult neurogenesis.

In conclusion, the present study revealed the c-jun protein expression pattern in the adult neurogenic region by immunohistochemistry and compared it with the embryonic brain. Our results suggest that c-jun might have distinct functions in embryonic and adult NPCs. In the embryonic NPCs, c-jun plays an important role in cell proliferation. On the other hand, c-jun activity might require proliferation, migration, and/or other phenomena in the sub-population of adult NPCs.

Funding:
This study was supported by JSPS KAKENHI Grant Number 25430044.

Conflict of interest:
The authors declare that they have no conflict of interest.

Figure legends

Fig. 1

c-jun protein is expressed in the embryonic and postnatal brain. (a–d) c-jun is expressed in the germinal layers of the E14 telencephalon. High magnification images in boxed areas are shown. c-jun immunoreactivity is more intense in the dorsal and ventral regions. Epi-fluorescent microscopy images are shown. (e–g) The majority of c-jun+ cells are MCM2+ proliferating cells at E14 (arrows). Single optical images of confocal microscopy are shown. (h–j) c-jun+ cells keep localizing in the germinal layers around the ventricle (dashed line). In the mantle layers, the number of c-jun+ cells increases with time. Light microscopy coronal-section images with chromogenic reaction from (h) E14, (i) 18, and (j) P2W brains are shown. CTX, cerebral cortex; GL, germinal layers; LV, lateral ventricle; ML, mantle layers; Str, striatum. Scale bars, 500 μm and 200 μm in d for low and high magnification images, respectively, 50 μm in g, 50 μm, 100 μm ,250 μm in j for h, i, j, respectively.

Fig. 2

c-jun protein is expressed in the adult SVZ. (a) Dense and basal c-jun immunoreactivity is detected in the adult SVZ (dashed line) and other parts of the brain (arrows). High magnification images in boxed areas are shown. Epi-fluorescent microscopy images are shown. (b–d) The majority, but not all, of c-jun+ cells in the adult SVZ are MCM2+ proliferating cells (arrows). Closed arrowheads and open arrowheads indicate c-jun single+ cells and MCM2 single+ cells, respectively. Ependymal cells are also c-jun+ but are not proliferating (dashed line). Single optical images of confocal microscopy are shown. (e) About 70% of c-jun+ cells are MCM2+ proliferating cells both in the embryonic germinal layers at E14 and in the adult SVZ. (f) Almost all MCM2+ proliferating cells in the embryonic germinal layers at E14 are c-jun+, but about half of them are c-jun+ in the adult SVZ. ns, not significant; **, $p < 0.01$. cc, corpus callosum. Scale bars, 500 μm and 100 μm in a for low and high magnification images, respectively, 50 μm in d.

Fig. 3

c-jun+ cells are migrating neuroblasts in the adult brain. (a–c) c-jun is not expressed in DCX+ cells. Arrowheads indicate c-jun single+ cells. (d–f) In the adult SVZ, virtually all c-jun+ cells are not GFAP+ (arrowheads), but there are only a few double+ cells (arrow). Many Mash1+ (g–i) and DCX+ (k–l) cells are c-jun+ (arrows). Closed

arrowheads and open arrowheads indicate c-jun single+ cells and marker single+ cells, respectively. Single optical images of confocal microscopy are shown. Scale bar, 50 μm in l.

Fig. 4

c-jun is expressed in the sub-population of tangentially migrating cells. (a–c) c-jun immunoreactivity decreases in the core of the OB. (d–f) c-jun+ cells are not DCX+ in the granule cell layer of the OB (open arrowheads), but they are NeuN+ mature neurons (arrows, g–i). Dashed lines indicate the SVZ, RMS, and core of the OB. Single optical images of confocal microscopy are shown. GCL, granule cell layer. Scale bar = 50 μm.

Fig. 5

The immunoreactivities of cell proliferation markers change along the SVZ-RMS-OB migration pathway in the adult brain. (a–c) MCM2 immunoreactivity is maintained even in the core of the OB. (d–f) Most BrdU+ cells are detected in the SVZ but not in other regions by a single injection and short-term survival protocol. Scale bar = 50 μm.

Fig. 6

c-jun protein expression is not affected by pilocarpine-induced status epilepticus. c-jun+ cells in the control (a and b) and SE (c and d) brains are shown. In the SE brain, induced c-jun protein expression is detected outside the SVZ (arrows). Epi-fluorescent microscopy images are shown. c-jun protein induction in the SE brain is not only in neurons (e) but also in astrocytes (f), oligodendrocytes (g) and microglia (h) in the striatum. Merged single optical images of confocal microscopy are shown. Co-localization of signals is shown as an orthogonal image. (i) The density of c-jun+ cells in the SVZ is not significantly different between the control and SE group. Scale bars = 500 μm and 100 μm in a for low and high magnification images, respectively, 25 μm in h.

Online Resource 1

c-jun+ cells are not migrating cells of the embryonic brain.

At E14, there is a scattering of c-jun+ cells lining the surface of the lateral ventricle that are DCX+ (arrows), and some of them are in telophase (double arrow). Single optical images of confocal microscopy are shown. Scale bar = 12.5 μm.

On line Resource 2

Status epilepticus enhances proliferation of adult NPCs. In the anterior part of the SVZ, larger number of BrdU+ cells are detected in the SE group (b) than in the control group (a) 1 week after induction of SE. Light microscopy images are shown. (c) The number of BrdU+ cells is compared (n = 3). *, $p < 0.05$. Scale bar = 200 μm.

References

Alfonso J, Penkert H, Duman C, et al (2015) Downregulation of Sphingosine 1-Phosphate Receptor 1 Promotes the Switch from Tangential to Radial Migration in the OB. J Neurosci 35:13659–13672.

Biebl M, Cooper CM, Winkler J, Kuhn HG (2000) Analysis of neurogenesis and programmed cell death reveals a self-renewing capacity in the adult rat brain. Neurosci Lett 291:17–20.

Blau L, Knirsh R, Ben-Dror I, et al (2012) Aberrant expression of c-Jun in glioblastoma by internal ribosome entry site (IRES)-mediated translational activation. Proc Natl Acad Sci U S A 109:E2875-84.

Bolognani F, Perrone-Bizzozero NI (2008) RNA-protein interactions and control of mRNA stability in neurons. J Neurosci Res 86:481–9.

Codega P, Silva-vargas V, Paul A, et al (2014) Prospective Identification and Purification of Quiescent Adult Neural Stem Cells from Their In Vivo Niche. Neuron 82:545–559.

Gleeson JG, Peter T L, Flanagan LA, et al (1999) Doublecortin is a microtubule-associated protein and is expressed widely by migrating neurons. Neuron 23:257–271.

Herdegen T, Leah JD (1998) Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expression by Jun, Fos and Krox, and CREB/ATF proteins. Brain Res Brain Res Rev 28:370–490.

Herdegen T, Waetzig V (2001) AP-1 proteins in the adult brain: facts and fiction about effectors of neuroprotection and neurodegeneration. Oncogene 20:2424–37.

Jiao X, Katiyar S, Willmarth NE, et al (2010) c-Jun induces mammary epithelial cellular invasion and breast cancer stem cell expansion. J Biol Chem 285:8218–26.

Kernie SG, Parent JM (2010) Neurobiology of Disease Forebrain neurogenesis after focal Ischemic and traumatic brain injury. Neurobiol Dis 37:267–274.

Kimelberg HK (2004) The problem of astrocyte identity. Neurochem Int 45:191–202.

Lalli G (2014) Extracellular signals controlling neuroblast migration in the postnatal brain. Adv Exp Med Biol 800:149–80.

Lee W-CA, Huang H, Feng G, et al (2006) Dynamic remodeling of dendritic arbors in GABAergic interneurons of adult visual cortex. PLoS Biol 4:e29.

Lledo P-M, Saghatelyan A (2005) Integrating new neurons into the adult olfactory bulb: joining the network, life-death decisions, and the effects of sensory experience. Trends Neurosci 28:248–54.

Lledo P, Alonso M, M G (2006) Adult neurogenesis and functional plasticity in neuronal circuits. Nat Rev Neurosci 7:179–193.

Lu D-Y, Leung Y-M, Cheung C-W, et al (2010) Glial cell line-derived neurotrophic factor induces cell migration and matrix metalloproteinase-13 expression in glioma cells. Biochem Pharmacol 80:1201–9.

Luhmann HJ, Fukuda A, Kilb W (2015) Control of cortical neuronal migration by glutamate and GABA. Front Cell Neurosci 9:4.

Maslov AY, Barone T a, Plunkett RJ, Pruitt SC (2004) Neural stem cell detection, characterization, and age-related changes in the subventricular zone of mice. J Neurosci 24:1726–33.

Mazzitelli S, Xu P, Ferrer I, et al (2011) The Loss of c-Jun N-Terminal Protein Kinase Activity Prevents the Amyloidogenic Cleavage of Amyloid Precursor Protein and the Formation of Amyloid Plaques In Vivo. J Neurosci 31:16969–16976.

Mellström B, Achaval M, Montero D, et al (1991) Differential expression of the jun family members in rat brain. Oncogene 6:1959–64.

Merkle FT, Mirzadeh Z, Alvarez-Buylla A (2007) Mosaic organization of neural stem cells in the adult brain. Science 317:381–4.

Merkle FT, Tramontin AD, García-Verdugo JM, Alvarez-Buylla A (2004) Radial glia give rise to adult neural stem cells in the subventricular zone. Proc Natl Acad Sci U S A 101:17528–17532.

Miyamoto A, Wake H, Ishikawa AW, et al (2016) Microglia contact induces synapse formation in developing somatosensory cortex. Nat Commun 7:12540.

Mori T, Buffo A, Götz M (2005) The novel roles of glial cells revisited: the contribution of radial glia and astrocytes to neurogenesis. Curr Top Dev Biol 69:67–99. d

Mori T, Wakabayashi T, Hirahara Y, et al (2012) Differential responses of endogenous adult mouse neural precursors to excess neuronal excitation. Eur J Neurosci 36:3184–93.

Ouafik L, Berenguer-Daize C, Berthois Y (2009) Adrenomedullin promotes cell cycle transit and up-regulates cyclin D1 protein level in human glioblastoma cells through the activation of c-Jun/JNK/AP-1 signal transduction pathway. Cell Signal 21:597–608.

Parent JM, Valentin V V, Lowenstein DH (2002) Prolonged seizures increase proliferating neuroblasts in the adult rat subventricular zone-olfactory bulb pathway. J Neurosci 22:3174–88.

Passegué E, Jochum W, Behrens A, et al (2002) JunB can substitute for Jun in mouse development and cell proliferation. Nat Genet 30:158–66.

Petreanu L, Alvarez-Buylla A (2002) Maturation and death of adult-born olfactory bulb granule neurons: role of olfaction. J Neurosci 22:6106–13.

Pinto JG a, Jones DG, Murphy KM (2013) Comparing development of synaptic proteins in rat visual, somatosensory, and frontal cortex. Front Neural Circuits 7:97.

Ponti G, Obernier K, Guinto C, et al (2013) Cell cycle and lineage progression of neural progenitors in the ventricular-subventricular zones of adult mice. Proc Natl Acad Sci U S A 110:E1045-54.

Pramparo T, Youn YH, Yingling J, et al (2010) Novel embryonic neuronal migration and proliferation defects in Dcx mutant mice are exacerbated by Lis1 reduction. J Neurosci 30:3002–12.

Raivich G, Behrens A (2006) Role of the AP-1 transcription factor c-Jun in developing, adult and injured brain. Prog Neurobiol 78:347–363.

Raivich G, Bohatschek M, Da Costa C, et al (2004) The AP-1 transcription factor c-Jun is required for efficient axonal regeneration. Neuron 43:57–67.

Spassky N, Merkle FT, Flames N, et al (2005) Adult ependymal cells are postmitotic and are derived from radial glial cells during embryogenesis. J Neurosci 25:10–8.

Takahashi T, Nowakowski RS, Caviness VS (1995) The cell cycle of the pseudostratified ventricular epithelium of the embryonic murine cerebral wall. J Neurosci 15:6046–6057.

Trachtenberg JT, Chen BE, Knott GW, et al (2002) Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex. Nature 420:788–94.

Wilkinson DG, Bhatt S, Ryseck RP, Bravo R (1989) Tissue-specific expression of c-jun and junB during organogenesis in the mouse. Development 106:465–71.

Wilsch-Bräuninger M, Florio M, Huttner WB (2016) Neocortex expansion in development and evolution - from cell biology to single genes. Curr Opin Neurobiol 39:122–32.

Zhang R, Zhang Z, Zhang C, et al (2004) Stroke transiently increases subventricular zone cell division from asymmetric to symmetric and increases neuronal differentiation in the adult rat. J Neurosci 24:5810–5.

Zhang RL, Zhang ZG, Roberts C, et al (2008) Lengthening the G(1) phase of neural progenitor cells is concurrent with an increase of symmetric neuron generating division after stroke. J Cereb Blood Flow Metab 28:602–11.

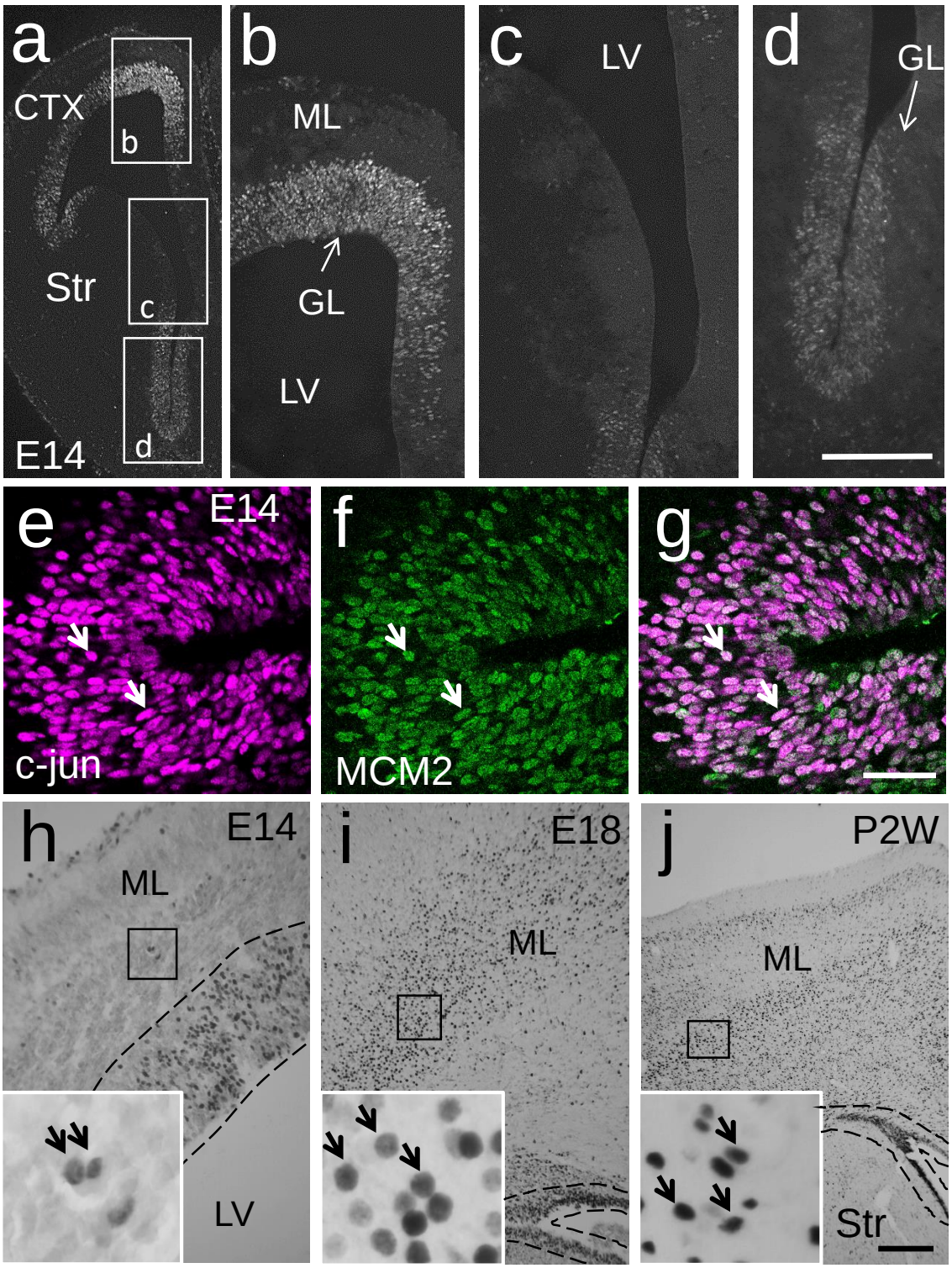

a
CTX
b
Str
c
d
E14
b
ML
GL
LV
c
LV
d
GL
e
E14
c-jun
f
MCM2
g
h
E14
ML
LV
i
E18
ML
j
P2W
ML
Str

Fig. 1

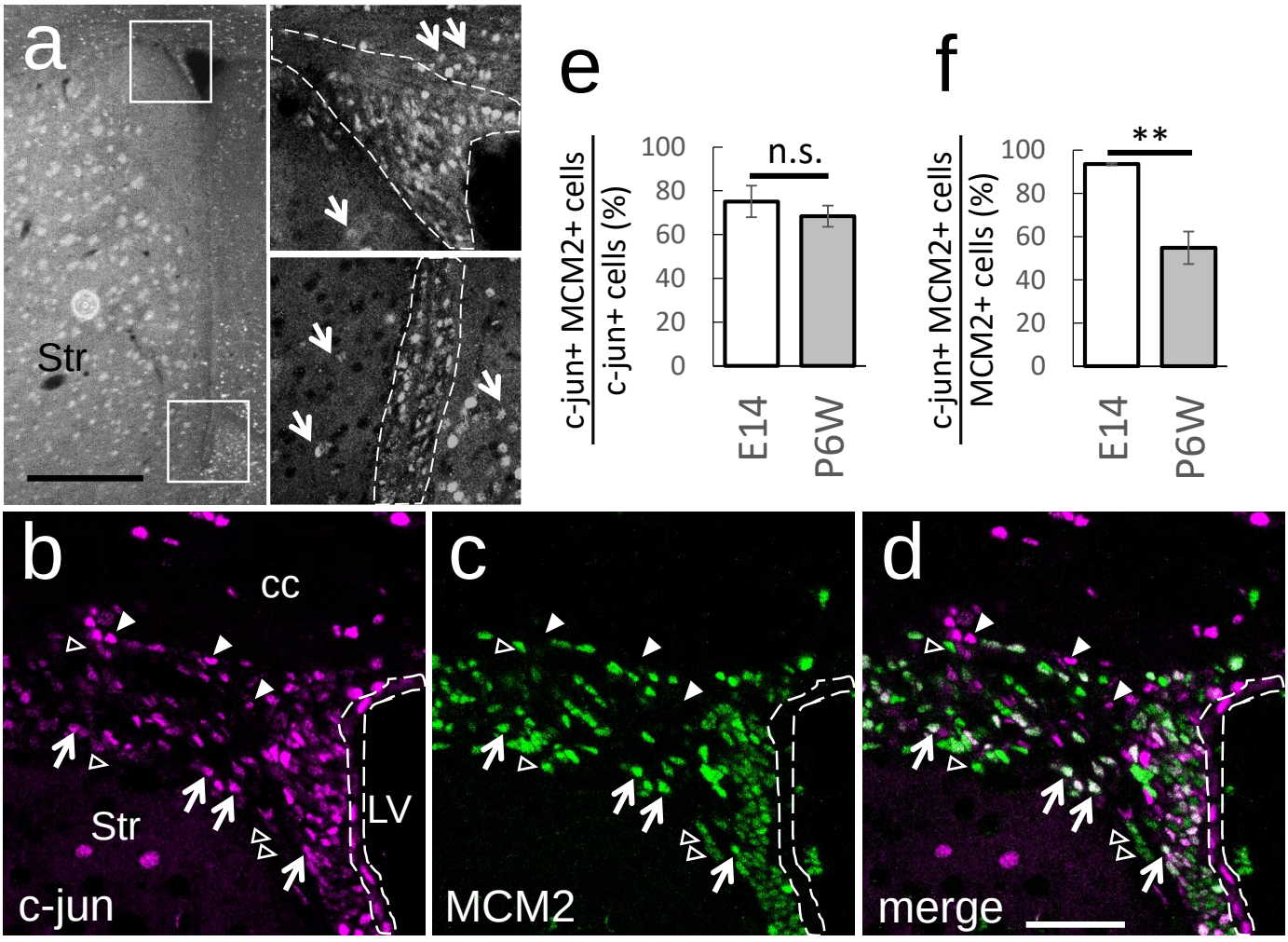

a
Str
e
c-jun+ MCM2+ cells
c-jun+ cells (%)
100
80
60
40
20
0
n.s.
E14
P6W
f
c-jun+ MCM2+ cells
MCM2+ cells (%)
100
80
60
40
20
0
**
E14
P6W
b
cc
Str
LV
c-jun
c
MCM2
d
merge

Fig. 2

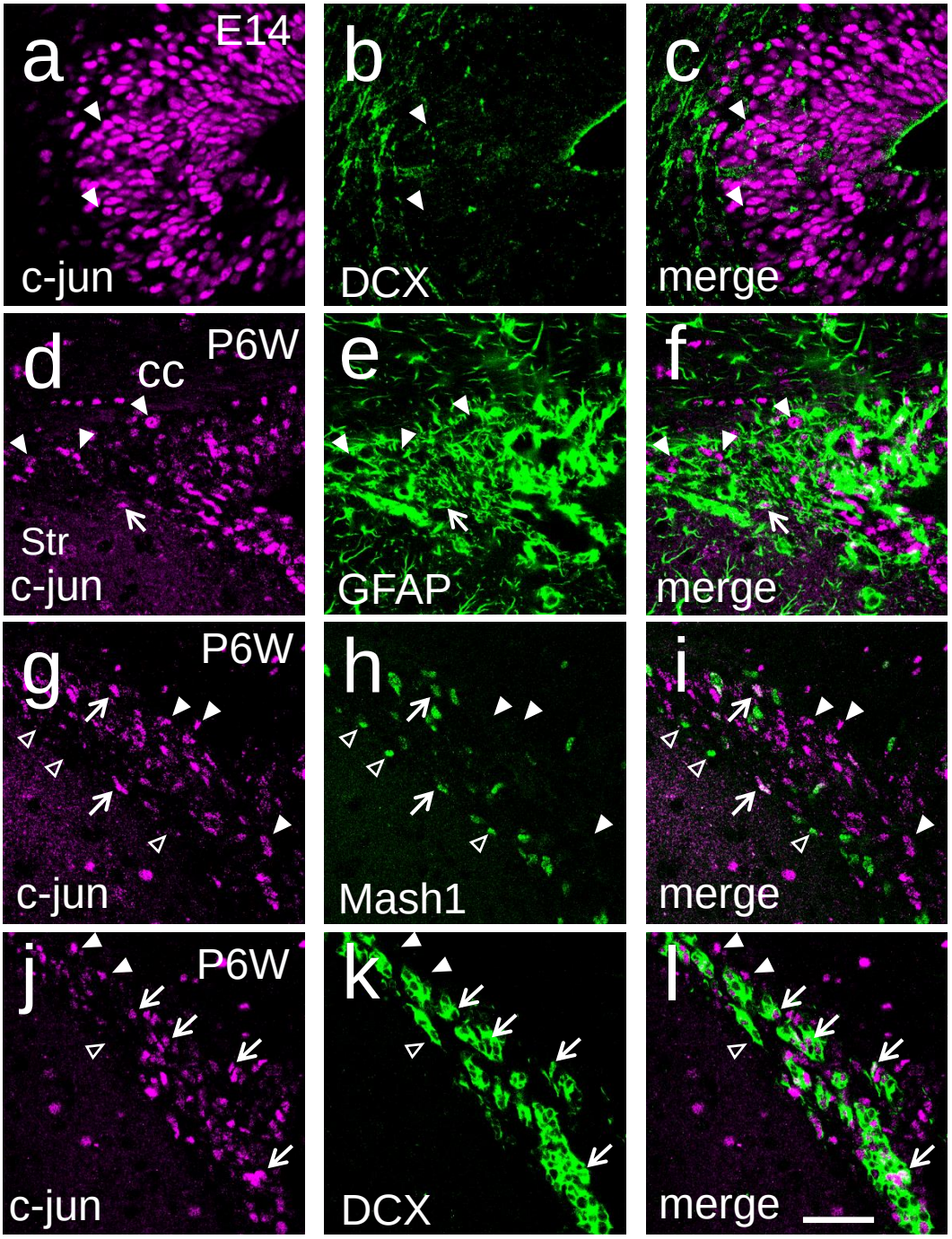

a
E14
c-jun
b
DCX
c
merge
d
P6W
cc
Str
c-jun
e
GFAP
f
merge
g
P6W
c-jun
h
Mash1
i
merge
j
P6W
c-jun
k
DCX
l
merge

Fig. 3

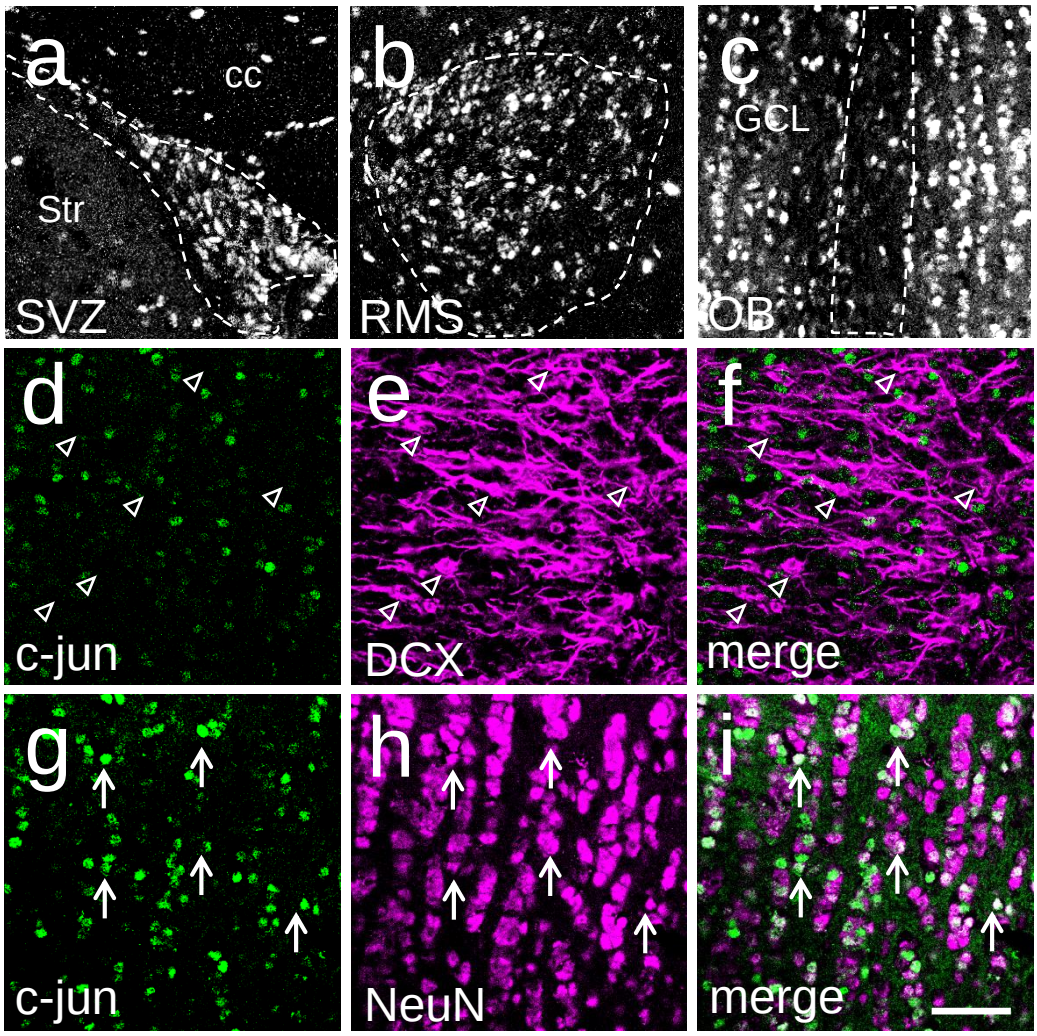

a
cc
Str
SVZ
b
RMS
c
GCL
OB
d
c-jun
e
DCX
f
merge
g
c-jun
h
NeuN
i
merge

Fig. 4

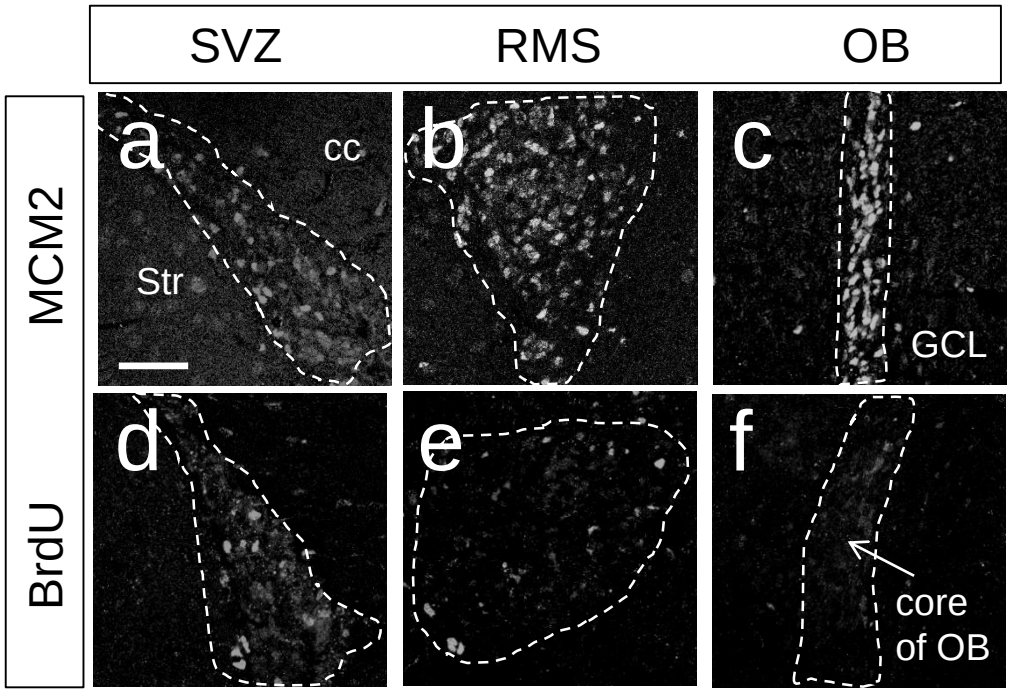


Fig. 5

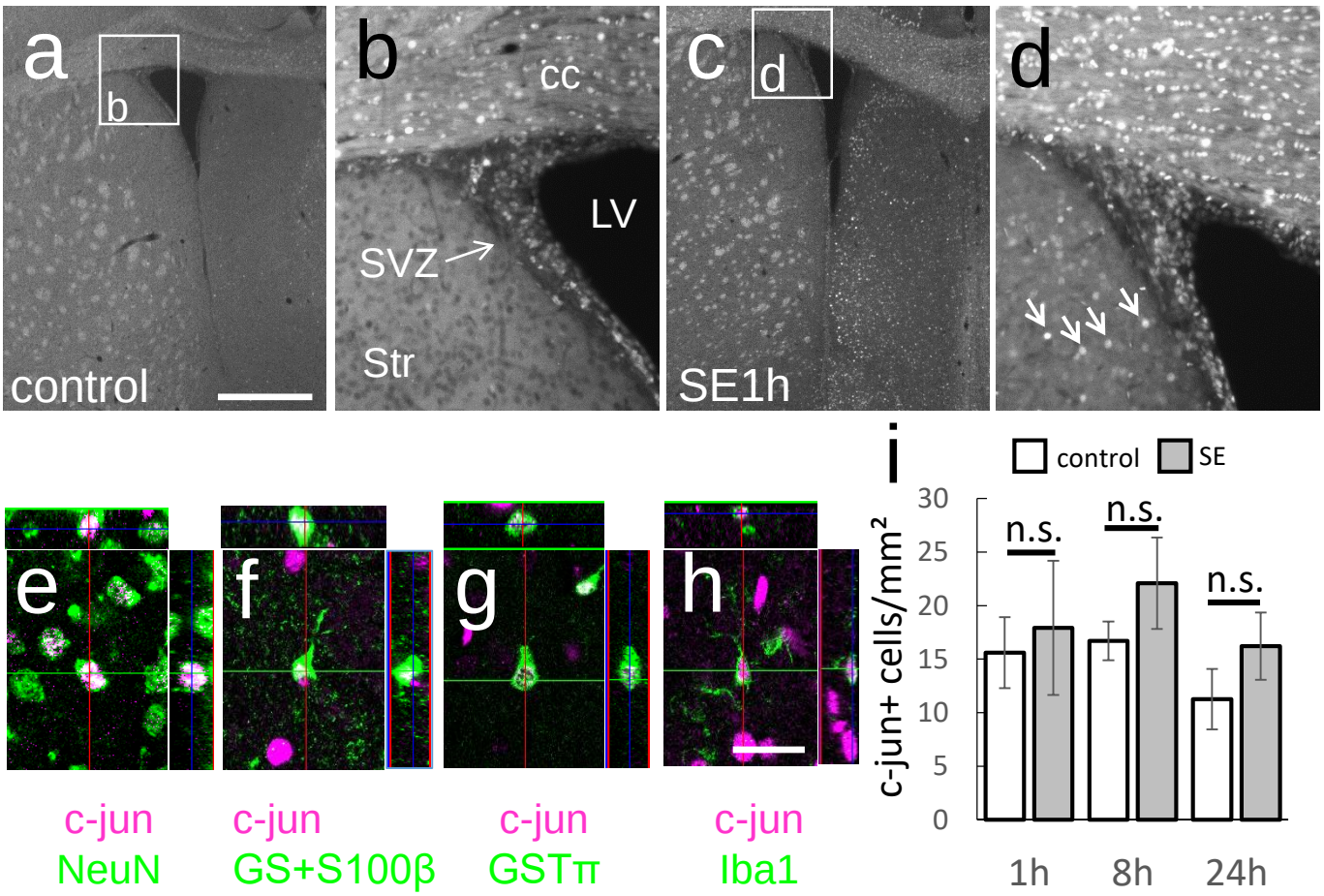
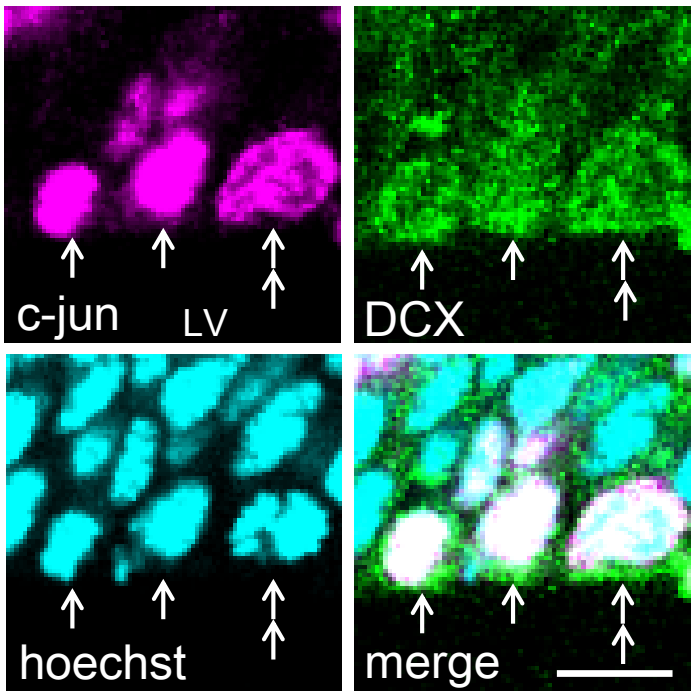
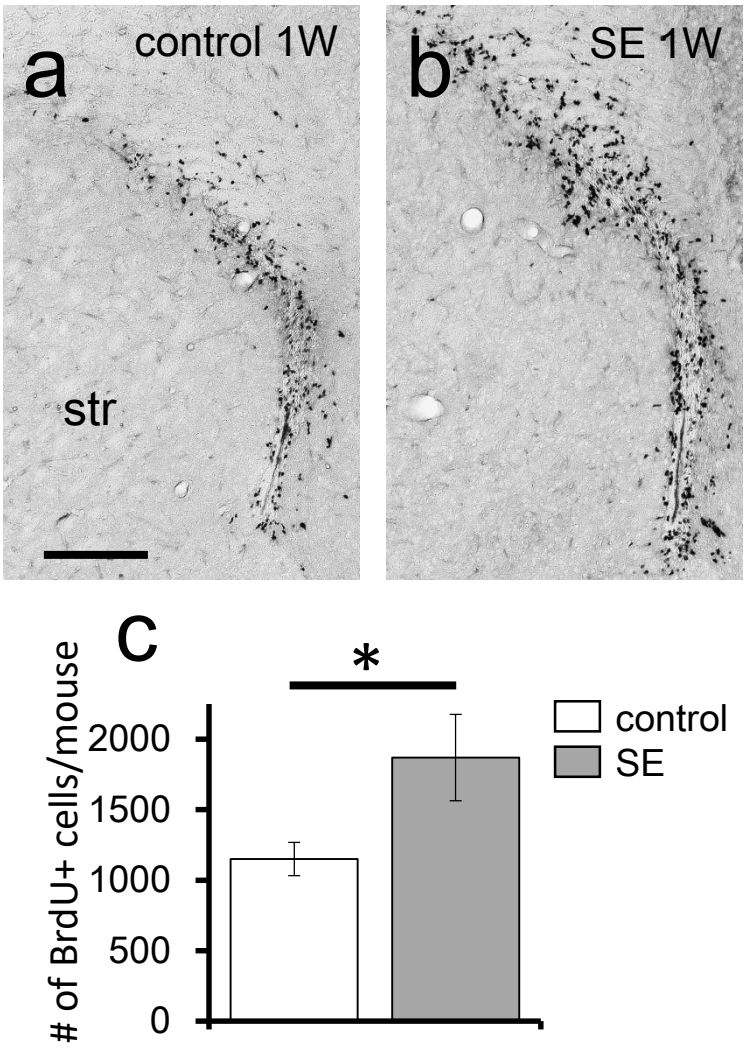

a
b
control
b
cc
LV
SVZ
Str
c
d
SE1h
d
e
f
g
h
c-jun
NeuN
c-jun
GS+S100β
c-jun
GSTπ
c-jun
Iba1
i
control
SE
c-jun+ cells/mm²
30
25
20
15
10
5
0
n.s.
n.s.
n.s.
1h
8h
24h

Fig. 6



Online Resource 1



Online Resource 2