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# Selective induction of ΔFosB in the brain after transient forebrain ischemia accompanied by an increased expression of galectin-1, and the implication of ΔFosB and galectin-1 in neuroprotection and neurogenesis

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## Abstract

**Transient forebrain ischemia causes selective induction of ΔFosB, an AP-1 (activator protein-1) subunit, in cells within the ventricle wall or those in the dentate gyrus in the rat brain prior to neurogenesis, followed by induction of nestin, a marker for neuronal precursor cells, or galectin-1, a β-galactoside sugar-binding lectin. The adenovirus-mediated expression of FosB or ΔFosB induced expression of nestin, glial fibrillary acidic protein and galectin-1 in rat embryonic cortical cells. ΔFosB-expressing cells exhibited a significantly higher survival and proliferation after the withdrawal of B27 supplement than the control or FosB-expressing cells. The decline in the ΔFosB expression in the survivors enhanced the MAP2 expression. The expression of ΔFosB in cells within the ventricle wall of the rat brain also resulted in an elevated expression of nestin. We therefore conclude that ΔFosB can promote the proliferation of quiescent neuronal precursor cells, thus enhancing neurogenesis after transient forebrain ischemia.**

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**Keywords:** AP-1; galectin-1; brain ischemia; neurogenesis; neuronal precursor

**Abbreviations:** AP-1, activator protein-1; AraC, 1-β-D-arabinofuranosylcytosine; DG, dentate gyrus; EGFP, enhanced green fluorescence protein; GCL, granule cell layer; GFAP, glial fibrillary acidic protein; IRES, internal ribosome entry site; LV, lateral ventricle; PEI, polyethylenimine; SGZ, subgranular zone; SHR, spontaneously hypertensive rat; SVZ, subventricular zone; Tet-Off system, inducible off control system of gene expression by tetracycline; VO, vessel occlusion; WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2*H*-tetrazolium

## Introduction

In mammals, the regulation of the cell fate to either proliferate, differentiate, arrest cell growth or initiate programmed cell death is the most fundamental mechanism for maintaining normal cell function and tissue homeostasis. Under multiple signaling pathways, Jun and Fos family proteins play important roles as components of the AP-1 (activator protein-1) complex[1,2] to regulate the transcription of various genes involved in cell proliferation, differentiation and programmed cell death.[3,4]

Among the four members of *fos* family genes (*c-fos, fosB, fra-1, fra-2*), only the *fosB* gene forms two mature mRNAs, *fosB* and *Δ fosB*, by alternative splicing,[5] each of which encodes at least three polypeptides by an alternative initiation of translation, called FosB, Δ1FosB, Δ2FosB, and ΔFosB, Δ1ΔFosB, Δ2ΔFosB, respectively.[6,7] The proteins encoded by *Δ fosB* mRNA lack the C-terminal 101-amino-acid region of the proteins encoded by *fosB* mRNA, which contains the motifs responsible for the interaction with TATA-box binding protein (TBP) and TFIID complex and also for the repression of *c-fos* and *fosB* promoters.[5,8] As well as other Fos family proteins, *fosB* gene products form heterodimers with each of the Jun (c-Jun, JunB, JunD) proteins, thereby stimulating the DNA-binding activities. We have previously shown that the proteins encoded by *Δ fosB* mRNA, such as ΔFosB, suppress the Jun transcription-activating ability acting on AP-1-dependent promoters;[5] however, others have demonstrated the ability of ΔFosB to activate the transcription by AP-1.[6,9] The ectopic expression of ΔFosB in transgenic mice revealed that ΔFosB indeed either upregulates or downregulates the expression of subsets of genes in the brain.[10,11] It is thus likely that the *fosB* gene products, especially ΔFosB, play an important role in the modulation of the gene expression regulated by AP-1.

In most types of rodent tissue, the *fosB* expression is either absent or barely detectable, while a basal expression of *fosB* is detected in some neurons scattered throughout the cerebral cortex and the hippocampus.[12] We have shown that the expression of the *fosB* gene, as well as *c-fos* or *c-jun*, is highly induced in the dentate gyrus (DG) of the hippocampus prior to the delayed neuronal loss in the CA1 subfield after transient

forebrain ischemia.[13] It has also been well established that neurogenesis can be induced in the adult mammalian brain after several forms of brain damage, such as seizure and transient ischemia, and it is restricted to specific regions such as the subventricular zone (SVZ) and DG of the hippocampus.[14–17] We previously found that ΔFosB, and to a lesser extent FosB, triggers one round of proliferation in the quiescent rat embryo cell lines rat 3Y1 and rat 1a followed by a different cell fate such as morphological alteration or delayed cell death, respectively.[18–22] We have also shown that the expression of galectin-1, one of the major $\beta$-galactoside sugar-binding lectins, is induced by ΔFosB in those cells, and it is required for the proliferative activation of quiescent rat 1a cells by ΔFosB,[21] thus indicating that galectin-1 is one of the functional targets of ΔFosB to modulate the cell fate, thus suggesting that ΔFosB, together with galectin-1, may play a critical role in determining the cell fate observed in the damaged brain.

In the present study, we found the expression of ΔFosB but not FosB to be selectively induced in cells within the ventricle wall, which also highly expressed nestin, a marker for neuronal precursor cells, as well as in the DG and CA1 subfield after transient forebrain ischemia in the rat brain. In a rat embryonic cortical cell culture, adenovirus-mediated expression of ΔFosB, and to a lesser extent FosB, promoted survival of nestin- and/or glial fibrillary acidic protein (GFAP)-positive cells after withdrawal of B27 trophic support, and also tended to induce their selective proliferation. The expression of ΔFosB in cells within the ventricle wall of the rat brain also resulted in an elevated expression of nestin. Furthermore, we demonstrated that the expression of galectin-1 is induced by FosB or ΔFosB in the embryonic cortical cells as well as in the hippocampus after transient forebrain ischemia.

## Results

### Expression of ΔFosB in brain after transient forebrain ischemia accompanied by the insult-induced neurogenesis

We previously reported the FosB and ΔFosB expression in hippocampus to be persistently elevated 2–48 h after transient forebrain ischemia produced by the four-vessel occlusion (4-VO) method, prior to the delayed CA1 neuronal loss in male Wister rats.[13] In the present study, we applied the 2-VO method to spontaneously hypertensive rats (SHR) in order to induce transient forebrain ischemia with a simple operation.[23] The CA1 neuronal loss was apparent 2–7 days after ischemic insult (Figure 1a and f). We then examined the FosB and ΔFosB expression in this model using two different antibodies against the *fosB* gene products. In the control rats, a weak immunoreactivity to the FosB(102) antibody, which recognizes both FosB and ΔFosB, was detected in some neurons scattered throughout the brain cortex and in the hippocampus (Figure 1b–e). At 2 days after the ischemic insult, there was a marked increase in the number of neurons that displayed a strong FosB(102) immunoreactivity in the entire region of the hippocampus (Figure 1g–j). Especially in the DG, the extent of immunoreactivity significantly varied from neuron to neuron in the granule cell layer (GCL) (Figure 1h). Cells with smaller nuclei exhibited a stronger immunoreactivity and such cells were more abundant in the subgranular zone (SGZ) of the DG and CA1 subfield. A strong FosB(102) immunoreactivity was also detected in the ependymal or subependymal cells within the lateral ventricle (LV) wall only after ischemic insult (Figure 1j). The brain sections shown in Figure 1 were subjected to double immunohistochemistry with anti-nestin sequentially. A moderate nestin immunoreactivity was detected throughout the cerebral cortex and in the hippocampus only after ischemic insult (Figure 1g–j). Some cells in SGZ with a strong FosB(102) immunoreactivity also exhibited a strong nestin immunoreactivity (Figure 1h), and such double-positive cells were also detected in the CA1 subfield (Figure 1i). Most of the ependymal or subependymal cells within the LV wall with FosB(102) immunoreactivity exhibited a strong nestin immunoreactivity (Figure 1j). Such increased immunoreactivities of FosB(102) and nestin were also apparent in cells within the third ventricle wall after ischemia (data not shown).

FosB(C) antibody against the C-terminal domain of FosB, which is missing in ΔFosB,[5] also exhibited an increased immunoreactivity in the hippocampus after the ischemic insult (Figure 2b and d). However, the FosB(C) immunoreactivity was detected in less than half of the hippocampal neurons, while FosB(102) immunoreactivity was detected in almost all neurons in the hippocampus (Figure 2a and c). There was no FosB(C) immunoreactivity detected in the ependymal or subependymal cells within the LV wall; however, most of them exhibited a strong nuclear FosB(102) immunoreactivity (Figure 2c and d). Most of the cells within the LV wall exhibited a strong nestin immunoreactivity in their cytoplasm after ischemic insult (Figure 2e).

As a result, we concluded that more than half of hippocampal neurons predominantly express ΔFosB, while the others express both FosB and ΔFosB. Furthermore, the ependymal or subependymal cells within the LV wall express high levels of nestin and ΔFosB but not FosB after the ischemic insult. It was noteworthy that the FosB(102) immunoreactivity in the brain apparently returned to the basal level 7 days after the insult (data not shown).

### Proliferative response in the brain after transient forebrain ischemia

In adult rodent brains, it has been established that DG in hippocampal formation and the SVZ are two major sites of high-density cell division,[15] and that such cell division is induced by various types of brain stress such as the ischemic insult.[14,16] To confirm whether such cell division occurs in the brain of SHR after the ischemic insult, bromodeoxyuridine (BrdU) was administered to the rats once a day for a week either with or without the insult. There was a significant increase in the number of BrdU-labeled cells in the hippocampus 7 days after the ischemic insult in comparison with the control brain (Figure 3a and f). BrdU-labeled cells were more abundant in the CA1 subfield and DG than in the CA3 subfield (Figure 3g–i). In this experiment, we observed a few foci of BrdU-positive cells in the cerebral cortex (Figure 3j), thus suggesting that the cerebral cortex of SHR exhibits hypervulnerability or enhanced proliferative response to the ischemic

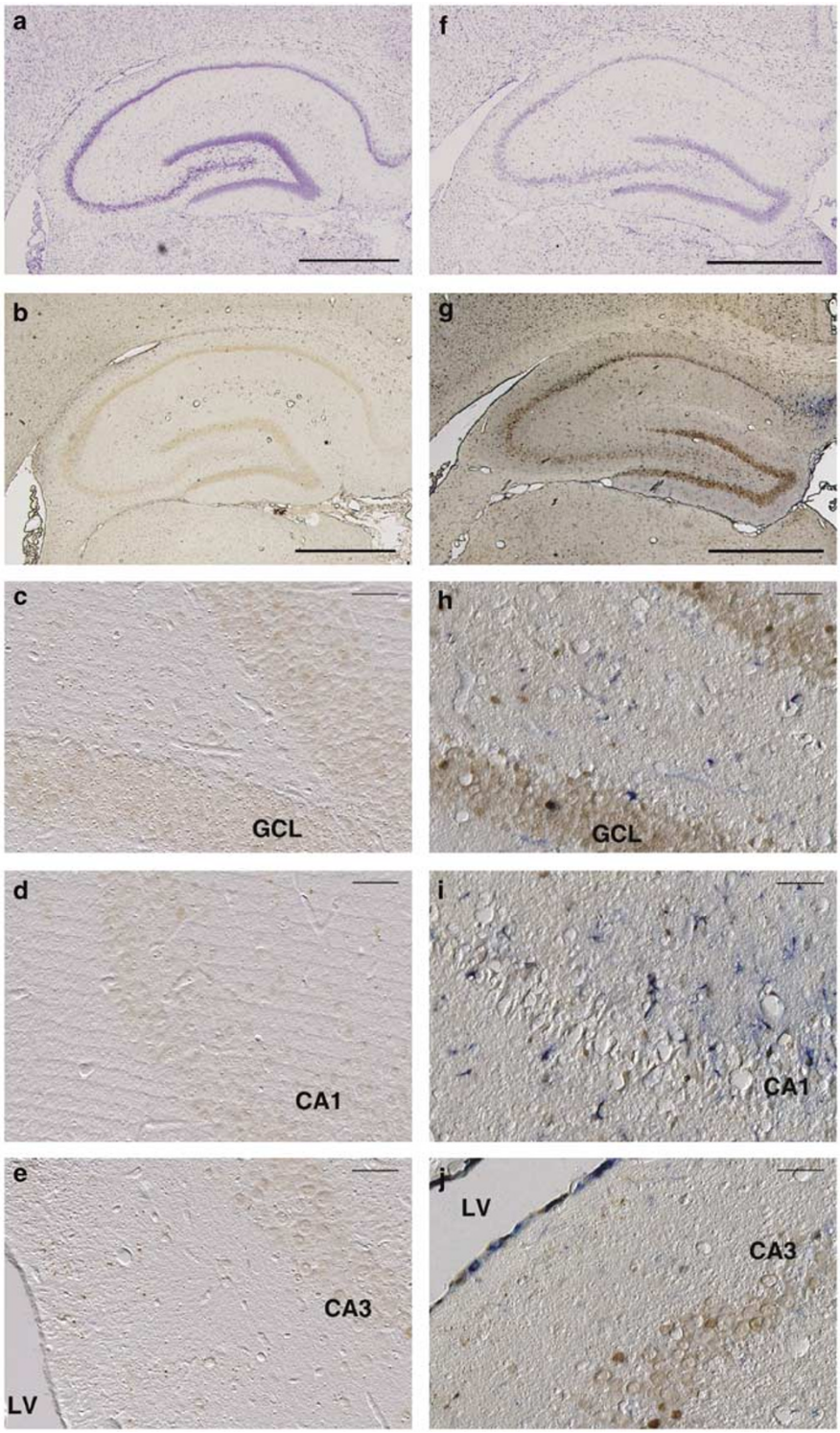


**Figure 1** Expression of *fosB* gene products in nestin-positive cells after transient forebrain ischemia. In the control animals (**a**–**e**), a weak FosB(102) immunoreactivity (**b**–**e**, brown) was detected in the neurons scattered within the cerebral cortex and hippocampus, and nestin immunoreactivity (**b**–**e**, blue) was barely detectable throughout the brain. At 2 days after 20 min of forebrain ischemia (**f**–**j**), neuronal loss in the CA1 subfield of the hippocampus was assessed by Cresyl violet staining (**a**, **f**). FosB(102) immunoreactivity was dramatically elevated in neurons within the cerebral cortex and hippocampus (**g**–**j**, brown), and nestin immunoreactivity was also moderately elevated throughout the brain (**g**–**j**, blue). In the DG (**c**, **h**), FosB(102) immunoreactivity was detected in most of the granule cells, but a few were positive for nestin immunoreactivity in the lesioned rat. In the CA1 subfield (**d**,**i**), double-positive cells with FosB(102) and nestin immunoreactivity were detected only in the lesioned rat. In the CA3 subfield (**e**, **j**), most of the pyramidal cells in the lesioned rats were positive for FosB(102) immunoreacitivity, but a few were positive for nestin immunoreactivity. In the ependymal cells within the LV wall (**e**, **j**), strong immunoreactivities both for FosB(102) and nestin were simultaneously detected only in the lesioned rat. Scale bars: 1 mm (**a**, **b**, **f**, **g**), 50 $\mu$m (**c**–**e**, **h**–**j**)

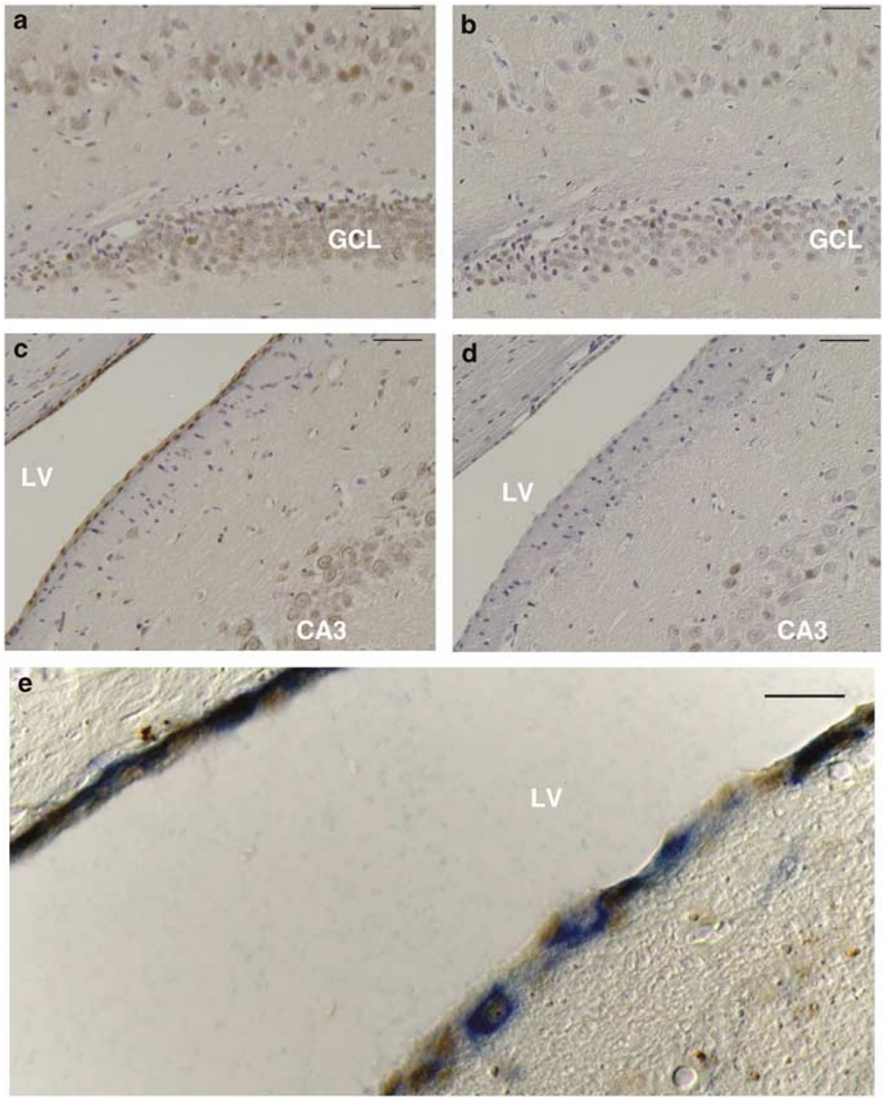


**Figure 2** ΔFosB is selectively expressed in the ependymal cells within the LV wall after transient forebrain ischemia. At 2 days after forebrain ischemia, FosB(102) immunoreactivity (**a**, **c**, **e**, brown) was detected in most of the dentate granule cells, CA3 pyramidal cells and ependymal cells within the LV wall. However, FosB(C) immunoreactivity was detected in some of the dentate granule cells or CA3 pyramidal cells but none of the ependymal cells within the LV (**b**, **d**). Most of the ependymal cells in the LV wall in the lesioned rat exhibited the nestin immunoreactivity in their cytoplasm (**e**, blue for nestin). As shown in Figure 11, both antibodies had the same immunoreactivity toward FosB, but anti-FosB(C) never reacted with ΔFosB. Scale bars: 50 $\mu$m (**a**–**d**), 20 $\mu$m (**e**)

insult. Proliferative responses in the brain are known to be accompanied by an increased expression of GFAP and nestin. We therefore examined the expression of nestin and GFAP in the brain with or without the ischemic insult (Figure 3k–v). In the ischemic brain, the number of nestin-expressing cells increased dramatically 7 days later, and their distribution was almost identical to that of BrdU-labeled cells in the hippocampus (Figure 3f–h, l, n and o). However, in the cerebral cortex, foci of BrdU-labeled cells were surrounded by nestin-positive cells (Figure 3f, j, l and p). Expression of GFAP in the lesioned rat was more significantly elevated surrounding the foci of BrdU-labeled cells in the cerebral cortex, as well as in the entire region of hippocampus, in comparison with that of nestin (Figure 3q–v).

As a result, we confirmed the expression of ΔFosB, and to a lesser extent FosB, to be persistently elevated in a subset of cells at the two sites of high-density cell division in the brain after the ischemic insult, thus suggesting that ΔFosB may play a role in the regulation of such cell division in response to brain insult.

## Adenovirus-mediated expression of *fosB* gene products in a rat embryonic cortical cell primary culture

The selective expression of ΔFosB in cells within the ventricle walls after ischemic insults strongly suggests that ΔFosB plays an important role during the stress response in the brain. Since *fosB* gene products possess the potential to initiate proliferation of quiescent cells,[18–22] we hypothesize that ΔFosB induces the proliferative activation of neuronal stem cells or precursor cells in the ischemic brain.

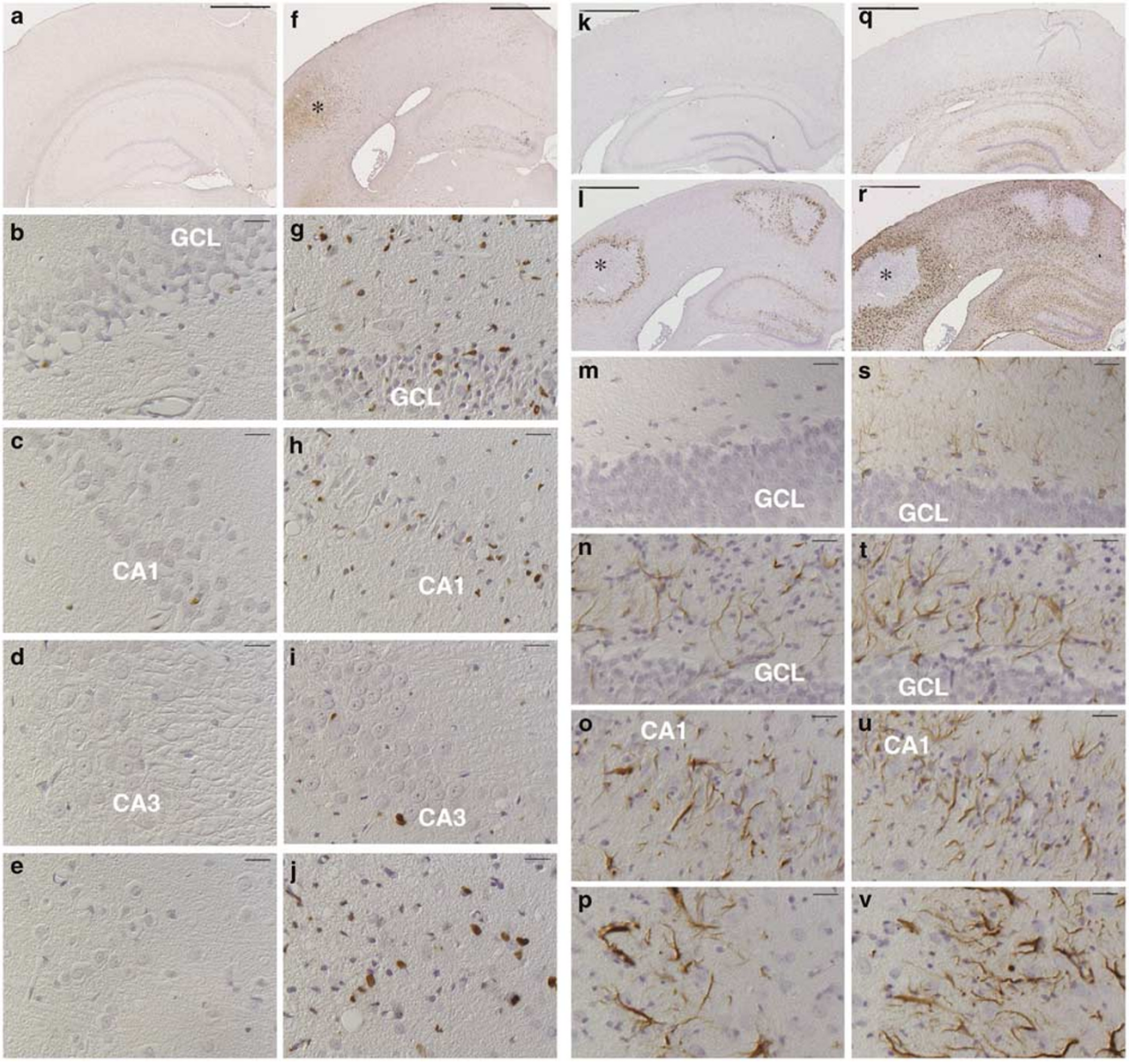


**Figure 3** Proliferative responses in brain after transient forebrain ischemia. BrdU (50 mg/kg) was administered intraperitoneally to the control rats (**a**–**e**, **k**, **m**, **q**, **s**) and to the rats with transient forebrain ischemia (**f**-**j**, **l**, **n**-**p**, **r**, **t**–**v**) once a day for a week. (**a**–**j**) Anti-BrdU immunostaining; (**k**–**p**) anti-nestin immunostaining; (**q**–**v**) anti-GFAP immunostaining. In the control animals (**a**, **k**), BrdU-labeled or nestin-positive cells were barely detectable throughout the brain. However, a weak GFAP immunoreactivity was detected in the hippocampus of the control animals (**q**). In the lesioned rats (**f**, **l**, **r**), the brain regions containing BrdU-labeled cells shown with asterisks were surrounded by nestin-positive and GFAP-positive cells. In the lesioned rats, a substantial number of cells in the DG (**g**, **n**, **t**), CA1 subfield (**h**, **o**, **u**) and subregions in the cerebral cortex (**j**, **p**, **v**), but not the CA3 subfield (**i**) exhibited strong BrdU, nestin and GFAP immunoreactivities. Scale bars: 1 mm (**a**, **f**, **k**, **l**, **q**, **r**), 20 $\mu$m (**b**–**e**, **g**–**j**, **m**–**p**, **s**–**v**)

To evaluate this hypothesis, we constructed adenovirus vector expressing *fosB* gene products under the control of the Tet-Off system (inducible off control system of gene expression by tetracycline). In order to visualize the adenovirus-infected cells, enhanced green fluorescence protein (EGFP) was coexpressed using an internal ribosome entry site (IRES) bicistronic expression system, which was placed downstream of FosB or ΔFosB cDNA. The expression of each *fosB* gene product was confirmed by Western blotting of rat 1a cells infected with each adenovirus together with Adeno-X Tet-Off virus expressing a tetracycline-controlled transactivator, tTA. Adenovirus carrying FosB cDNA produced 45, 35 and 30-kDa polypeptides, which most likely correspond to the polypeptides translated from the first, second and third methionine codons of FosB mRNA, while adenovirus carrying ΔFosB cDNA produced 35, 25 and 22-kDa polypeptides, corresponding to the translation products by the alternative translation initiation,[7] reacted with anti-FosB(102) (Figure 4a). Confocal laser-scanning fluorescence microscopy with the same antibody revealed that FosB or ΔFosB was expressed only in cells

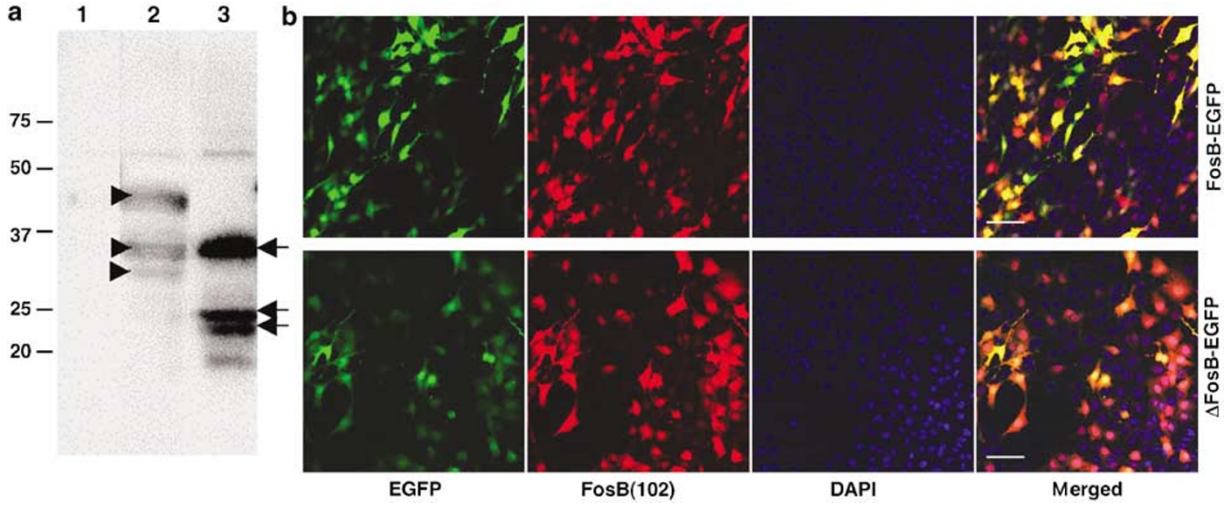


**Figure 4** Expression of FosB and ΔFosB by recombinant adenovirus. (**a**) Western blotting analysis. Cell lysates prepared from rat 1a cells infected with recombinant adenovirus encoding EGFP alone (lane 1), FosB-EGFP (lane 2) and ΔFosB-EGFP (lane 3), together with Adeno-X Tet-Off virus, were subjected to a Western blot analysis using FosB(102) antibody. The arrowheads indicate the alternative translation initiation products from *fosB* mRNA, and the arrows indicate those from *ΔfosB* mRNA. (**b**) Laser-scanning fluorescence microscopy with anti-FosB(102). Rat 1a cells infected with each recombinant adenovirus (FosB-EGFP, ΔFosB-EGFP), as indicated on the right side, were fixed, and the nuclei were counterstained with DAPI. Confocal images were obtained by laser-scanning fluorescence microscopy after reaction with anti-FosB(102). Each fluorescence is shown in green (EGFP fluorescence), red (FosB(102) immunofluorescence) and blue (DAPI). Merged: the merged view of the three fluorescences. Scale bars, 100 $\mu$m

infected with the recombinant adenoviruses encoding FosB or ΔFosB, and both were localized in the cytoplasm as well as in the nuclei (Figure 4b). The expression of FosB or ΔFosB from the adenoviral vector was completely abolished in the presence of a minimum of 5 ng/ml doxycycline (data not shown).

We next applied the adenoviruses to rat embryonic cortical cells to induce the expression of FosB and ΔFosB in neuronal precursor cells. We prepared embryonic cortical cells from the brain cortex together with the hippocampus of 18-day-old rat embryos; thus the embryonic cortical cells used in this study contain the neuronal precursor cells derived from the SVZ and hippocampus. Adenovirus-mediated expression of *fosB or ΔfosB* mRNA in rat embryonic cortical cells was confirmed by semiquantitative RT-PCR analyses (Figure 5a and b), thus indicating that each recombinant adenovirus produces only the *fosB* or *ΔfosB* transcript, respectively, and that their levels were almost equivalent.

Since it has been shown that neuronal precursor cells, such as radial glias or radial cells, which occupy the cortical and ventricular surfaces, express the highest level of an adenovirus receptor, CAR, adenovirus was proved to have a high probability of infecting those cells.[24,25] Therefore, rat embryonic cortical cells were infected with each recombinant adenovirus at MOI = 1, and EGFP-positive cells, which represented adenovirus-infected cells, were mostly MAP2 or $\beta$-tubulin III-negative (data not shown), thus indicating that few mature neurons were infected with recombinant adenoviruses. The morphology of cells expressing EGFP was likely to be different from each other (Figure 5c–e). More than half of all EGFP-positive cells without FosB or ΔFosB expression exhibited a typical morphology of astrocytes, namely the irregular and roughly star-shaped cell bodies. However, EGFP-positive cells expressing FosB or ΔFosB tended to exhibit a unipolar or bipolar shape.

In a rat embryonic cortical cell primary culture, immunohistochemistry with three Jun-specific antibodies revealed the expression of FosB and ΔFosB to be accompanied by increased expression of c-Jun, JunB and JunD (Figure 5c–e). FosB and ΔFosB were largely detected in the cytoplasm; however, an increased JunD immunoreactivity was apparently detected in the nuclei of cells expressing FosB or ΔFosB among the three Jun proteins. Semiquantitative RT-PCR analyses showed the *JunB* mRNA levels, to a lesser extent *c-jun*, to increase more than 2.5-fold in FosB-expressing embryonic cortical cells, but no such increase was seen in

**Figure 5** Adenovirus-mediated expression of FosB and ΔFosB in embryonic cortical cells and the coexpression of Jun proteins. (**a**) Schematic representation of *fosB* and *ΔfosB* mRNAs. The closed regions indicate the common sequences for the two mRNAs while the open region indicates the 140-base sequence that is alternatively spliced out in *ΔfosB* mRNA. The primers used to amplify the common region (*fosB-5′*) for *fosB* and *ΔfosB* mRNA are shown by arrows and those used to amplify the sequence (*fosB-3′*) within the 140-base sequence are shown by arrowheads. Specific TaqMan probes for the *fosB-5′* and *fosB-3′* sequences are shown by a gray and hatched box, respectively. (**b**) Expression of *fosB* and *ΔfosB* mRNAs in rat embryonic cortical cells after the infection of adenovirus encoding EGFP alone, FosB-EGFP and ΔFosB-EGFP. Rat embryonic cortical cells infected with each recombinant adenovirus (EGFP, FosB-EGFP, ΔFosB-EGFP), as indicated on the bottom, were cultured for 2 days after the withdrawal of B27 trophic support. The relative amount of each RT-PCR product for *Gapdh* product was determined by monitoring the PCR product in real time. Gray bar: PCR products from the common region (*fosB*-5′); hatched bar: PCR products from the primers specific for *fosB* mRNA (*fosB*-3'). (**c**–**e**) The coexpression of Jun proteins in rat embryonic cortical cells induced by FosB or ΔFosB. Rat embryonic cortical cells infected with each recombinant adenovirus (EGFP alone, FosB-EGFP, ΔFosB-EGFP), as indicated on the left side, were cultured for 3 days, and then were fixed and subjected to immunofluorescent microscopy with FosB(102) and each Jun-specific antibody (**c**: c-Jun; **d**: JunB; **e**: JunD). Each fluorescence is shown in green (EGFP fluorescence), red (FosB(102) immunofluorescence) and blue (Jun immunofluorescence). Merged: the merged view of the three fluorescences. Scale bars, 50 $\mu$m

ΔFosB-expressing embryonic cortical cells, in comparison to the cells expressing only EGFP (Table 1). On the other hand, *JunD* mRNA levels in embryonic cortical cells expressing FosB and ΔFosB decreased to 83 and 73% of the levels in EGFP-expressing cells, respectively. The mRNA levels of *Cdk5* and *GluR2* whose expressions were reported to be induced in the striatal neurons by ΔFosB,[10,11] did not increase at all, or rather slightly decreased, especially in embryonic

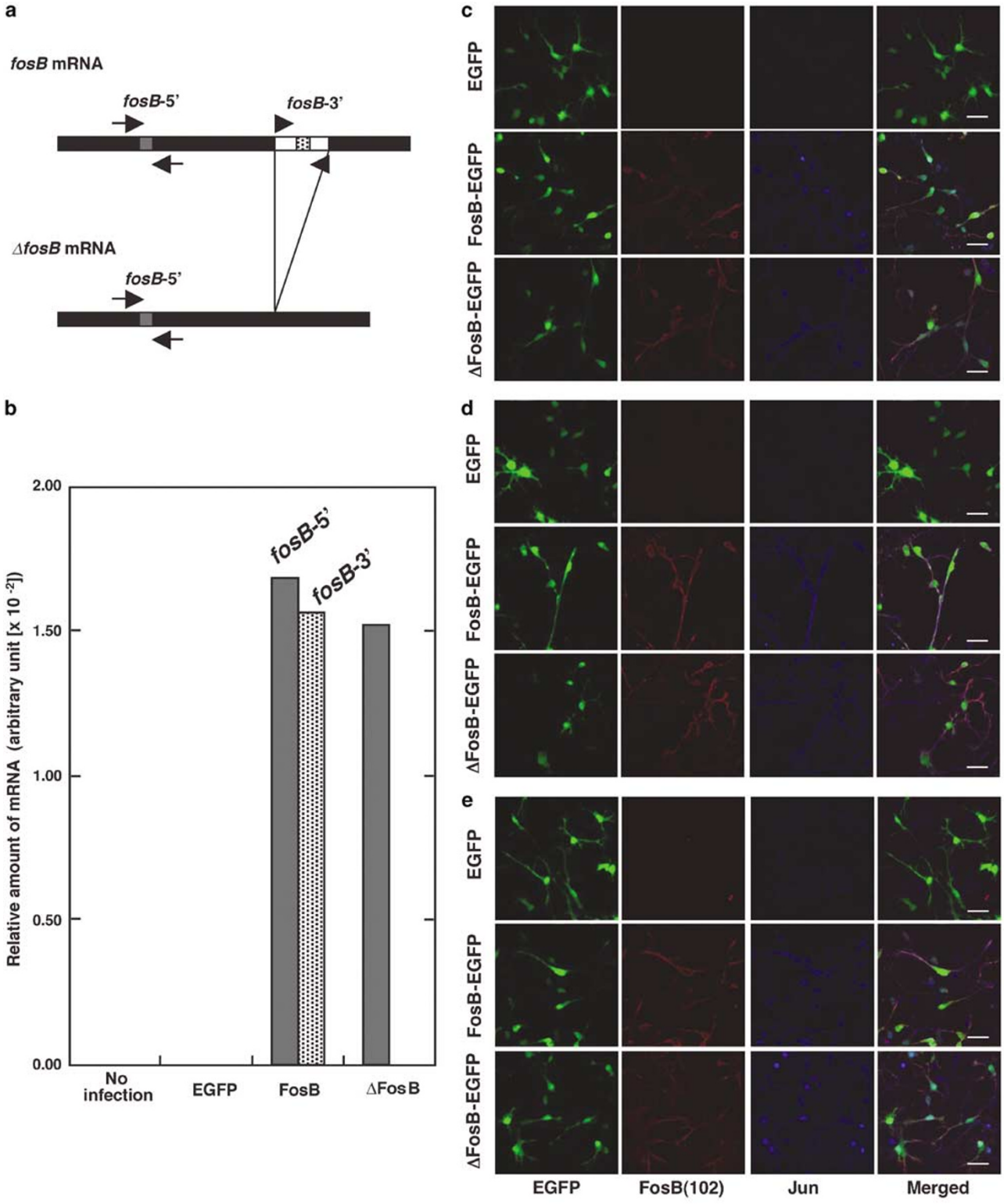


**Figure 5**

**Table 1** Altered gene expression in embryonic cortical cells expressing FosB or ΔFosB

| Gene | Relative level of transcript in cortical cells expressing[a] | | |
|---|---|---|---|
| | EGFP | FosB | ΔFosB |
| c-*Jun* | 1.00±0.10 | 1.17±0.05 | 1.00±0.18 |
| *JunB* | 1.00±0.09 | 2.65±0.22 | 1.14±0.04 |
| *JunD* | 1.00±0.04 | 0.83±0.01 | 0.73±0.02 |
| *Mmp3* | ND | ND | ND |
| *Cdk5* | 1.00±0.07 | 1.00±0.09 | 0.94±0.02 |
| *GluR2* | 1.00±0.10 | 0.95±0.04 | 0.90±0.05 |
| *Gfap* | 1.00±0.05 | 1.68±0.11 | 1.43±0.01 |
| *Nestin* | 1.00±0.07 | 1.56±0.10 | 1.66±0.27 |

Rat embryonic cortical cells were infected with each recombinant adenovirus encoding EGFP alone, FosB-EGFP or ΔFosB-EGFP, and then were cultured for 4 days in the presence of B27 supplement. At 2 days after the culture medium was changed to a Neurobasal medium lacking B27 supplement, total RNA were prepared and subjected to RT-PCR and amplification was monitored in real time. Experimental values obtained for each transcript were divided by the values for *Gapdh* mRNA in the same preparation. Level of each transcript was normalized to that in cells expressing EGFP alone. [a]Mean values with S.D. from three independent PCR are shown. ND: not detected

cortical cells expressing ΔFosB. Regardless of either FosB or ΔFosB expression, no detectable *Mmp3* mRNA was observed, whose expression is known to be upregulated by FosB but not ΔFosB in rat 1a cells as one of the AP-1-responsive genes.[18]

## ΔFosB stimulates the proliferation of the embryonic cortical cells after the withdrawal of B27 trophic support

To examine the biological significance of FosB or ΔFosB expression in the embryonic cortical cells, viability of adenovirus-infected cells was determined after the withdrawal of the B27 trophic support, which is essential for their maintenance from the cultured medium.[26,27] As shown in Figure 6a, the culture that received adenovirus expressing EGFP alone exhibited a slight increase of cells within the first 2 days after B27 withdrawal and thereafter a decreased cell viability was observed, as determined by 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium (WST-8) formazan assay. In contrast, the cultures that received adenovirus expressing FosB or ΔFosB exhibited more growth than the control cells within the first 2 days, and showed a decreased viability on the 4th day. At 1 week after B27 withdrawal, only ΔFosB-expressing cells recovered significantly and increased their viability.

In order to identify cells that survived after B27 withdrawal, the cells were examined under fluorescent microscopy and the number of EGFP-positive cells was determined. As shown in Figure 6b, the number of EGFP-positive cells in culture infected with adenovirus expressing EGFP alone decreased continuously after B27 withdrawal and only 30% of the EGFP-positive cells survived on the 8th day after B27 withdrawal. The expression of FosB maintained the number of EGFP-positive cells for the first 4 days; however, the number

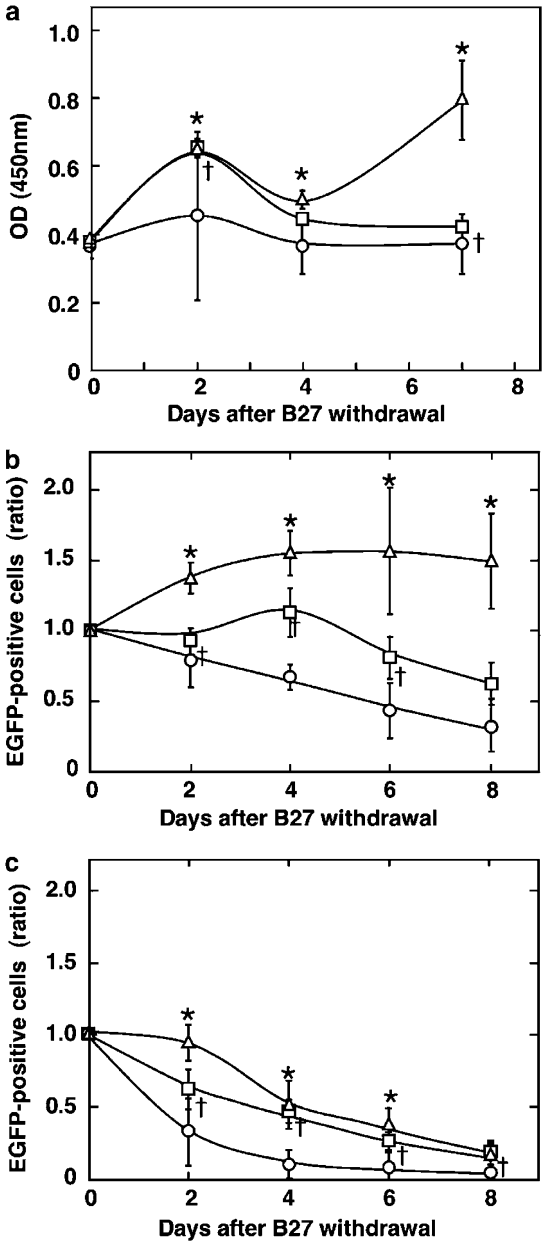


**Figure 6** ΔFosB, and to a lesser extent FosB, induced proliferation of rat embryonic cortical cells in the absence of B27 supplement. (**a**) Cell viability. Rat embryonic cortical cells were infected with each recombinant adenovirus, and then were cultured for 3 days in the presence of B27 supplement. After the culture medium was changed to Neurobasal medium lacking B27 supplement, cell viability was determined using WST-8 assay as described in Materials and Methods, every other day. (**b**) Proliferation of EGFP-positive cells. After the culture medium was changed to a Neurobasal medium lacking B27 supplement, the number of EGFP-positive cells in the same field was counted every other day. (**c**) Survival of EGFP-positive cells. After the infection of each recombinant adenovirus, the rat embryonic cortical cells were cultured for 3 days in the presence of 10 $\mu$M AraC and B27 supplement. After the culture medium was changed to a Neurobasal medium lacking B27 supplement, the number of EGFP-positive cells in the same field was counted every other day. Circle: EGFP alone; squares: FosB-EGFP; triangles: ΔFosB-EGFP. The data are shown with the means±S.D. (four independent experiments). The cell number was normalized to that of day 0. Statistical significance ($P<0.05$) is indicated by † for FosB-EGFP and * for ΔFosB-EGFP, in comparison with EGFP alone

decreased thereafter and about 60% of EGFP-positive cells survived on the 8th day. In contrast, embryonic cortical cells infected with adenovirus expressing ΔFosB increased the number of EGFP-positive cells more than 1.5 times within 4 days after B27 withdrawal, and most EGFP-positive cells survived thereafter.

We next examined the effect of the cytidine analog, 1-$\beta$-D-arabinofuranosylcytosine (AraC) on the embryonic cortical cells. AraC inhibits DNA replication, and thus only postmitotic cells, such as neurons, can survive. Most of the EGFP-positive cells in culture that received adenovirus expressing EGFP alone disappeared within the first 4 days after B27 withdrawal, while the expression of ΔFosB, and to a lesser extent FosB, significantly improved the survival of EGFP-positive cells (Figure 6c).

It is likely that ΔFosB, and to a lesser extent FosB, stimulates embryonic cortical cell proliferation even in the absence of B27 trophic support. We next examined whether ΔFosB or FosB stimulates DNA synthesis in embryonic cortical cells. In the presence of BrdU, embryonic cortical cells were infected with each adenovirus, and were cultured for 3 days. The incorporation of BrdU into nuclear genomes was monitored by immunofluorescence microscopy (Figure 7 and Table 2). In the culture receiving adenovirus expressing EGFP alone, none of the EGFP-positive cells incorporated BrdU. On the other hand, about 6% of the EGFP-positive cells in culture receiving adenovirus expressing FosB or ΔFosB were strongly labeled with anti-BrdU antibody. We thus concluded that FosB and ΔFosB stimulate the cell proliferation of embryonic cortical cells, and that ΔFosB, and to a lesser extent FosB, promotes cell survival after B27 withdrawal.

Because there were quite a few BrdU-labeled and EGFP-negative cells around EGFP-positive cells, some factor(s) secreted from cells expressing FosB or ΔFosB may promote their proliferation (Figure 7).

## Coexpression of galectin-1 and nestin in the embryonic cortical cells expressing FosB or ΔFosB, as well as in the brain after transient ischemia

In rat 1a cells, the ectopic expression of ΔFosB increased the expression of galectin-1, which was partly responsible for their proliferation induced by ΔFosB.[20,21] We examined the expression of galectin-1 in the cortical cells after FosB or ΔFosB expression, since galectin-1 is known to be a secretory

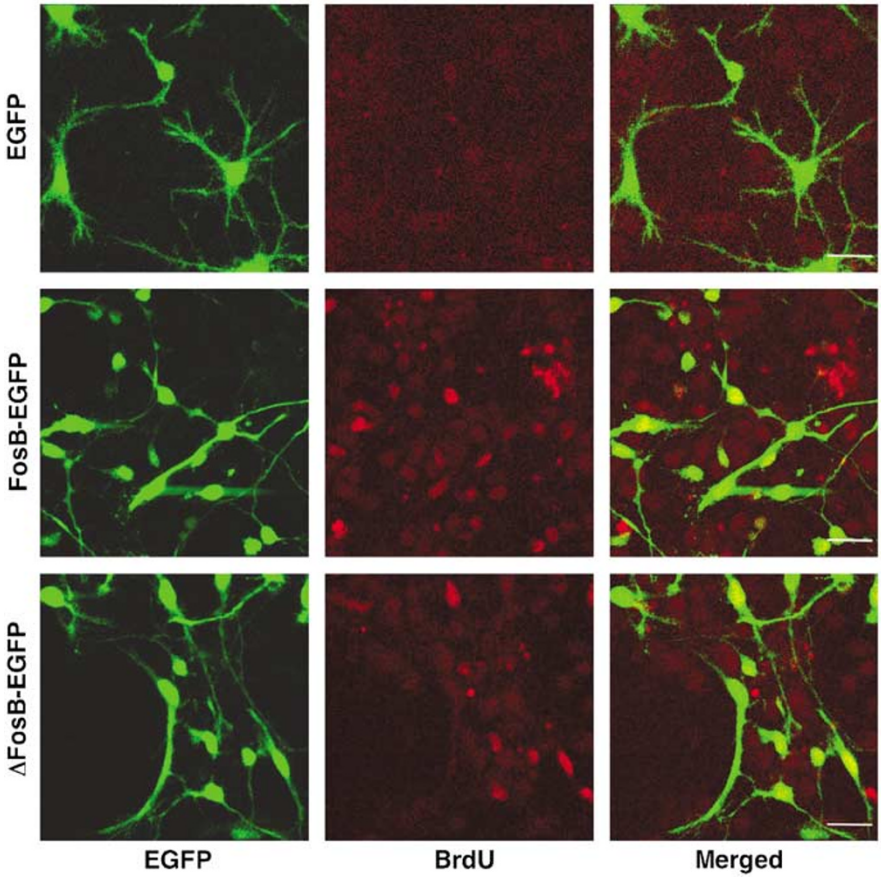


**Figure 7** DNA synthesis in adenovirus-infected rat embryonic cortical cells. Rat embryonic cortical cells were infected with each recombinant adenovirus encoding EGFP alone, FosB-EGFP and ΔFosB-EGFP, as indicated on the left side, and were cultured in the presence of B27 supplement and 10 $\mu$M BrdU for 3 days. The cells were fixed and subjected to immunofluorescence microscopy with anti-BrdU antibody. Green: EGFP fluorescence; red: anti-BrdU immunofluorescence; merged: the merged view of the two fluorescences. Scale bars, 50 $\mu$m

**Table 2** DNA synthesis in adenovirus-infected rat embryonic cortical cells

| Adenovirus | No. of EGFP$^+$ cells | No. of BrdU$^+$ cells | % BrdU$^+$ cells |
|---|---|---|---|
| EGFP | 116 | 0 | 0 |
| FosB-EGFP | 177 | 11 | 6.2 |
| ΔFosB-EGFP | 180 | 10 | 5.6 |

Rat embryonic cortical cells were infected with recombinant adenoviruses and then were cultured in the presence of B27 supplement and 10 μM BrdU for 3 days. The cells were fixed and subjected to immunofluorescence microscopy with anti-BrdU antibody as shown in Figure 7. The number of cells in areas of the same size was counted. Among EGFP-positive cells, only cells exhibiting a strong BrdU immunoreactivity were counted as BrdU$^+$

factor that can regulate cell fate such as proliferation or apoptosis.[22] As shown in Figure 8a, galectin-1 was barely detected in the embryonic cortical cells expressing EGFP alone; however, a significantly higher level of galectin-1 as well as nestin was detected in the embryonic cortical cells expressing FosB or ΔFosB. We next examined the effects of galectin-1 on proliferation of the embryonic cortical cells. In the presence of a low dose of recombinant galectin-1α (50 pg/ml), the proliferation of the embryonic cortical cells was slightly, but significantly, promoted in comparison to that in the absence of galectin-1α (Figure 8b), thus indicating that galectin-1α, as one type of secretory factor, has a potential to promote the proliferation of embryonic cortical cells. Interestingly, a higher dose of galectin-1α (5 μg/ml) or galectin-1β (50 pg/ml or 5 μg/ml, data not shown), which is known to be a variant form of galectin-1,[22] exhibited a much weaker effect.

We next examined the expression of galectin-1 in the rat brain after transient forebrain ischemia by confocal laser-scanning fluorescence microscopy (Figure 8c). In the control brain, the expression of neither galectin-1 nor nestin was detectable, while a significantly higher level of galectin-1 was detected in the hippocampus after ischemia, where the nestin expression also showed a dramatic increase. Most of the nestin-positive cells in the hippocampus expressed galectin-1, while the nestin-positive cells within the ventricle wall did not express galectin-1 (data not shown).

## Expression of neuronal markers in the rat embryonic cortical cells expressing FosB or ΔFosB

In order to identify the cell type whose proliferation was stimulated by FosB or ΔFosB, we examined the expression of various neuronal markers such as MAP2, β-tubulin III, nestin and GFAP in the rat embryonic cortical cells maintained in the absence of B27 supplement. The majority of the embryonic cortical cells were MAP2- or β-tubulin III-positive matured neurons; however, they were exclusively EGFP-negative (data not shown). In contrast, EGFP-positive cells were largely nestin-positive, and some of them were also GFAP-positive, regardless of the FosB or ΔFosB expression (Figure 9 and Table 3). EGFP-positive cells in the culture that received adenovirus expressing EGFP alone exhibited the typical morphology of astrocytes with GFAP expression, namely the irregular and roughly star-shaped cell bodies with weaker immunoreactivity to anti-nestin. A quarter of nestin-positive cells were GFAP-negative and they were somehow morphologically different from the rest (Figure 9). In contrast, EGFP-positive cells expressing FosB exhibited mostly a bipolar shape with a stronger immunoreactivity to anti-nestin, and less than a quarter of them exhibited immunoreactivity to anti-GFAP. We found more abundant EGFP-positive cells in the culture receiving adenovirus expressing ΔFosB in comparison to those expressing FosB, and most of them also exhibited a strong immunoreactivity to anti-nestin (Table 3). ΔFosB-expressing cells exhibiting immunoreactivity both to anti-nestin and anti-GFAP possessed dendrite-like structures with elongated neurite-like structures, while those with a single immunoreactivity to anti-nestin exhibited a bipolar shape. As shown in Table 1, the mRNA levels of *Gfap* and *Nestin* increased from 43 to 68% in embryonic cortical cells expressing either FosB or ΔFosB, in comparison to cells expressing only EGFP.

## ΔFosB can maintain the immature property of neuronal precursor cells and the decline of ΔFosB may play an important role in neuronal maturation

Rat embryonic cortical cells expressing FosB or ΔFosB in the absence of B27 supplement, as shown in Figure 9, most likely proliferate neuronal precursor cells. To address this question, we turned either the FosB or ΔFosB expression off by adding doxycycline to the culture. We then monitored the expression of MAP2 in the EGFP-positive cells in the presence of B27 supplement. As shown in Figure 10, 4 days after the addition of doxycycline, MAP2-positive cells appeared only in the culture receiving adenovirus expressing ΔFosB but not FosB or EGFP alone (data not shown). Unfortunately, doxycycline also decreased the expression of EGFP; therefore, we could observe only a small number of EGFP-positive cells in the experiment. In the absence of doxycycline, EGFP-positive cells were more abundant but none of the EGFP-positive cells exhibited immunoreactivity to anti-MAP2 (Figure 10).

**Figure 8** The coexpression of galectin-1 and nestin in the embryonic cortical cells expressing FosB or ΔFosB as well as in the brain after transient forebrain ischemia. (**a**) Rat embryonic cortical cells were infected with each recombinant adenovirus encoding EGFP alone, FosB-EGFP and ΔFosB-EGFP, as indicated on the left side, and then were cultured for 4 days in the presence of B27 supplement. At 2 days after the culture medium was changed to a Neurobasal medium lacking B27 supplement, the cells were fixed and subjected to immunofluorescence microscopy. Green: EGFP fluorescence; red: nestin immunofluorescence; blue: GFAP immunofluorescence; merged: the merged view of the three fluorescences. Scale bars, 50 μm. (**b**) Galectin-1α promotes proliferation of the cortical cells *in vitro*. Rat embryonic cortical cells were cultured in the Neurobasal medium with B27 supplement in the absence (control) or presence of galectin-1α (50 pg/ml or 5 μg/ml). At 4 days later, the cell viability was determined using a WST-8 assay. $^*P<0.05$, compared with the control. (**c**) Galectin-1 is expressed in the hippocampus formation after transient forebrain ischemia. The brain sections were prepared from the rats 7 days after the transient forebrain ischemia, and then were subjected to confocal laser-scanning microscopy after a reaction with anti-rhGal-1 (red) or anti-nestin (green) with a proper Alexa-labeled second antibody. Galectin-1 and nestin were mostly colocalized in the hippocampus. Blue: DAPI; Merged: the merged view of the three fluorescences. Scale bars: 25 μm

### ΔFosB expression in cells within the ventricle wall induces the expression of nestin but not GFAP

In order to examine whether ΔFosB expression in the ependymal or subependymal cells within the ventricle wall alters their phenotype, recombinant adenovirus coding EGFP alone, FosB-EGFP or ΔFosB-EGFP was injected into the ventricle. As shown in Figure 11, we found EGFP-positive cells within some limited regions of the ventricle wall 2 days

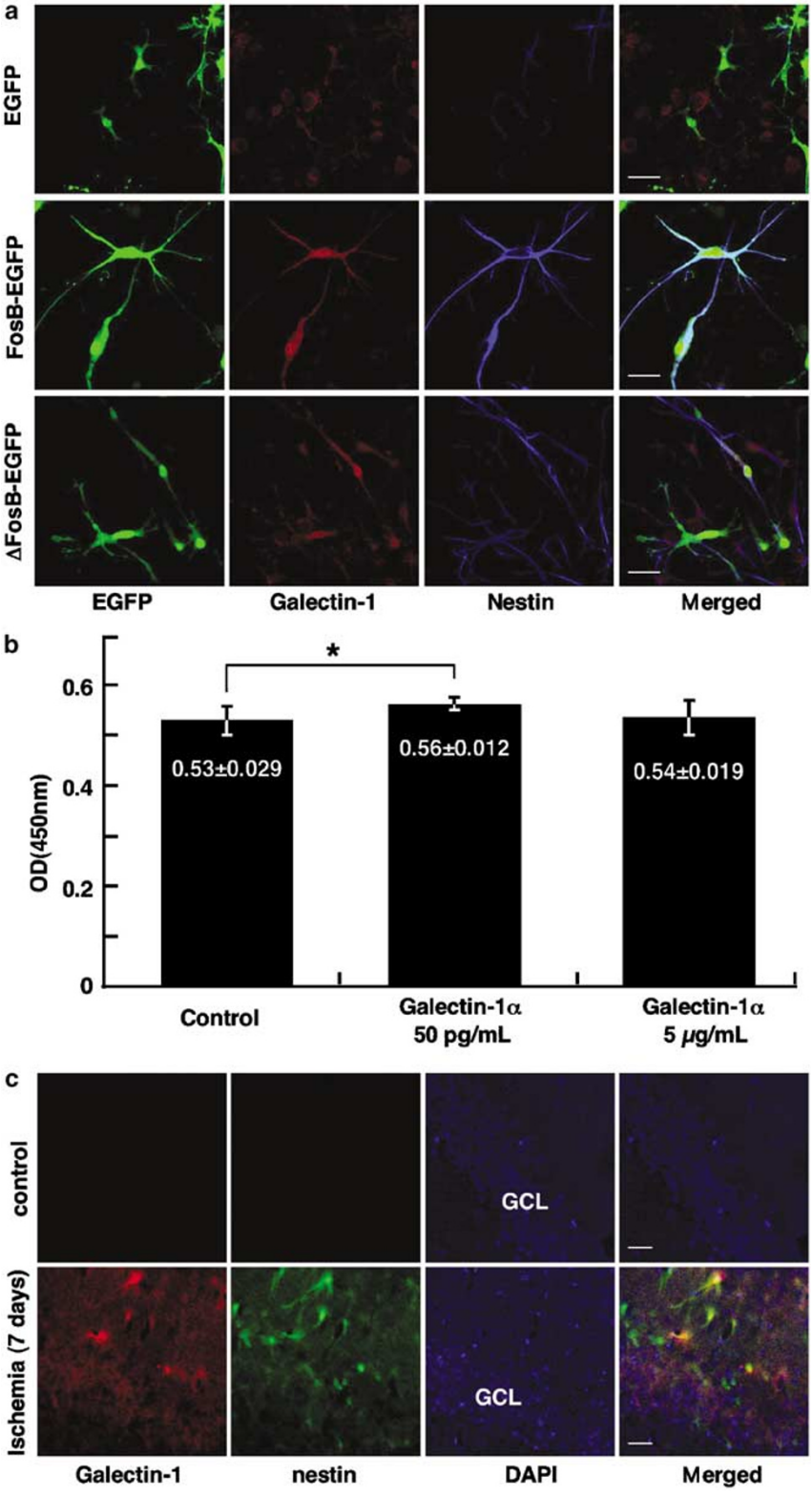


**Figure 8**

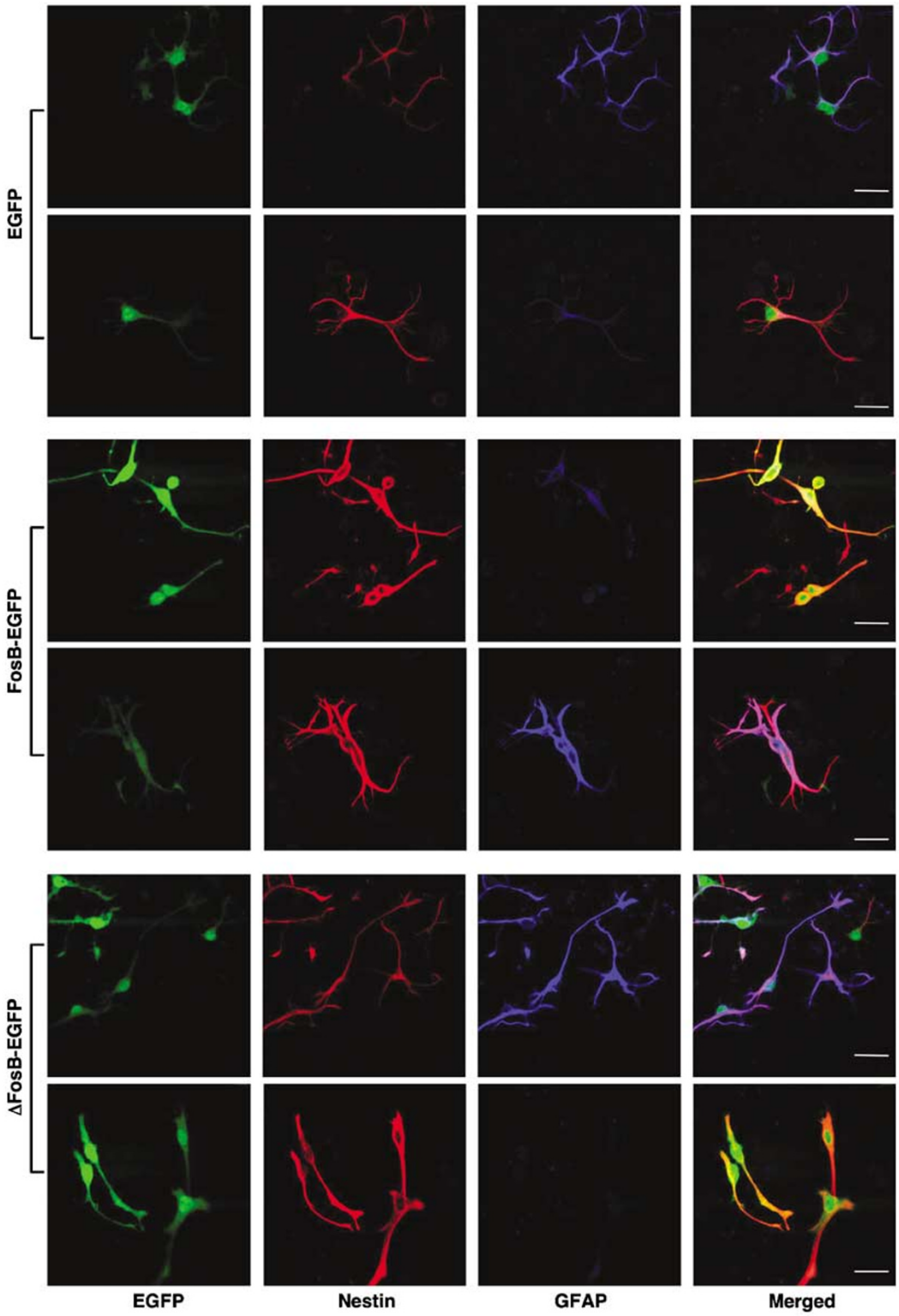


**Figure 9** Phenotypical changes of the rat embryonic cortical cells induced by FosB or ΔFosB expression. Rat embryonic cortical cells were infected with each recombinant adenovirus encoding EGFP alone, FosB-EGFP and ΔFosB-EGFP, as indicated on the left side, and then were cultured for 4 days in the presence of B27 supplement. At 2 days after the culture medium was changed to a Neurobasal medium lacking B27 supplement, the cells were fixed and subjected to immunofluorescence microscopy. Green: EGFP fluorescence; red: nestin immunofluorescence; blue: GFAP immunofluorescence; merged: the merged view of the three fluorescences. Scale bars, 50 $\mu$m

**Table 3** Expression of nestin and GFAP in adenovirus-infected rat embryonic cortical cells

| | Number of EGFP-positive cells | | | |
|---|---|---|---|---|
| **Adenovirus** | **Nestin$^+$/GFAP$^+$** | **Nestin$^+$/GFAP$^-$** | **Nestin$^-$/GFAP$^+$** | **Nestin$^-$/GFAP$^-$** |
| EGFP | 15 | 5 | 2 | 1 |
| FosB-EGFP | 5 | 16 | 0 | 0 |
| ΔFosB-EGFP | 17 | 23 | 1 | 5 |

Rat embryonic cortical cells were infected with each recombinant adenovirus encoding EGFP alone, FosB-EGFP or ΔFosB-EGFP, and then were cultured for 4 days in the presence of B27 supplement. At 2 days after the culture medium was changed to a Neurobasal medium lacking B27 supplement, the cells were fixed and subjected to immunofluorescence microscopy for nestin and GFAP. The number of cells in areas of the same size was counted

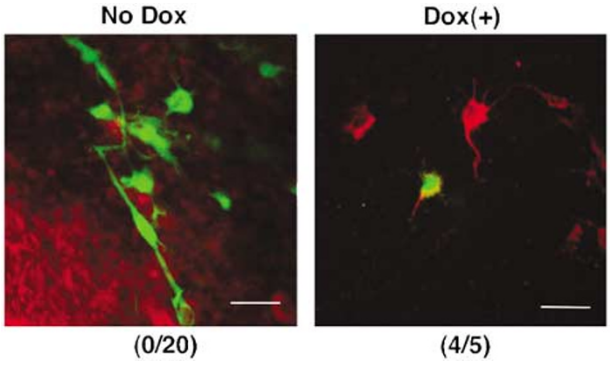


**Figure 10** Expression of neural marker in rat embryonic cortical cells after the expression of ΔFosB was shut-off. Rat embryonic cortical cells infected with the recombinant adenovirus coding ΔFosB-EGFP were cultured for 2 days in the presence of B27 supplement, and then the cells were maintained in Neurobasal medium lacking B27 supplement for 13 days. Doxycycline (Dox (+), final 400 ng/ml) was added to half of the cultures, and the rest was maintained without doxycycline (No dox). After 4 days, the cells were fixed and subjected to immunofluorescence microscopy with anti-MAP2 antibody. Red: MAP2 immunofluorescence; green: EGFP fluorescence. The merged views are shown. In parentheses, total numbers of MAP2-positive and EGFP-positive cells observed in the experiment are shown (MAP2/EGFP). Scale bars, 50 $\mu$m

after infection, and mostly only ependymal or subependymal cells expressed EGFP. The cells infected with adenovirus coding FosB-EGFP exhibited immunoreactivities both for anti-FosB(102) and anti-FosB(C), while those with adenovirus coding ΔFosB-EGFP exhibited immunoreactivity only for anti-FosB(102) but not anti-FosB(C), and neither immunoreactivity was detected in those with adenovirus coding EGFP alone (Figure 11). As a result, we reproduced the specific expression of ΔFosB in cells within the ventricle wall, as we observed in the rats after ischemic insult.

We next examined the expression of nestin and GFAP in the adenovirus-infected cells, as shown in Figure 12. The nestin expression level was low in normal ependymal or subependymal cells and in those infected with adenovirus coding EGFP alone. However, the nestin immunoreactivity in cells expressing FosB and ΔFosB increased significantly in comparison to those infected with adenovirus coding EGFP alone or adjacent EGFP-negative cells. Interestingly, the cells expressing ΔFosB maintained a nonpolar shape, while those expressing FosB tended to show a polar shape. GFAP immunoreactivity in cells expressing FosB or ΔFosB was not elevated in comparison to nestin; however, the cells neighboring the subependymal layer showed an increased GFAP immunoreactivity in comparison to the control. Again, these observations may suggest that some secretary factor(s) from FosB- or ΔFosB-expressing cells may promote the proliferation of neuronal precursor cells.

As seen in the embryonic cortical cell culture, the expression of ΔFosB in cells within the ventricle wall also increased the expression of nestin but not that of GFAP.

## Discussion

Our major conclusion in the present study is that the expression of ΔFosB was induced in cells within the ventricle wall as well as in the hippocampus prior to neurogenesis after transient forebrain ischemia; that the expression of ΔFosB in cells within the ventricle wall resulted in an elevated expression of nestin; that ΔFosB triggered the proliferation of neuronal progenitor-like cells; and that the decline in ΔFosB expression plays important roles in promoting the maturation of neurons.

In the brain of adult rats, the expression of *fosB* gene products, FosB and ΔFosB, is very low; however, various types of brain insult, such as transient forebrain ischemia or excitotoxicity, result in a significant induction of their expression in the hippocampus prior to neuronal loss, as well as other AP-1 proteins such as c-Fos and c-Jun or JunB.[13] Among them, the induction of c-Fos expression is very rapid and transient and its level returns to basal level within a couple of hours after transient forebrain ischemia, while the levels of FosB and ΔFosB or c-Jun expression persistently elevated 2–48 h after the insult. The level of JunB expression is slowly elevated 12–24 h after transient forebrain ischemia, while the level of JunD expression is almost constant either with or without the insult. Since the phosphorylation of c-Jun by Jun amino-terminal kinases (JNK) such as JNK3 plays a key role in apoptosis or neurodegeneration, the induced expression of these AP-1 proteins after transient forebrain ischemia has been implicated in the neuronal loss caused by insult.[28,29]

In the present study, we found the expression of ΔFosB but not that of FosB to be selectively induced in cells within the ventricle wall as well as in the hippocampus 2 days after the transient forebrain ischemia, and that these cells also exhibited a markedly elevated expression of nestin, which is a class IV intermediate filament protein and a marker for neuronal precursor cells in the adult brain.[30,31] At 7 days after ischemia, although the ΔFosB expression was as low as at the basal level, the nestin-positive cells were enriched in the outer

boundary zones to the ischemic core in the cerebral cortex as well as in the CA1 and DG subfields of the hippocampus, where BrdU-positive cells were also enriched. We further observed an increased expression of galectin-1 in the nestin-positive cells in the hippocampus but not within the ventricle wall, at 7 days after ischemia. Since ΔFosB as well as FosB increased the expression of both galectin-1 and nestin in the embryonic cortical cells, ΔFosB induced in the hippocampus after ischemia is therefore likely responsible for the increased expression of galectin-1, and we are now confirming this possibility using *fosB*-null mice.

We previously demonstrated that an artificial expression of ΔFosB, and to a lesser extent FosB, triggers DNA replication and cell division in quiescent fibroblast cells in the absence of serum.[18–21] Furthermore, neurogenesis in the adult mammalian brain has been proven to occur in the SVZ and DG of the hippocampus, and it is likely to be promoted by stress such as ischemic insult.[14–17] Therefore, our observation strongly suggests that the ischemia-induced expression of ΔFosB in cells within the ventricle wall or neuronal precursor cells in DG promotes the proliferation or migration of such neuronal precursor cells to the damaged area in the brain to replace the damaged neurons. Furthermore, our data suggest that the decline in the ΔFosB expression following the proliferation is essential for maturation of neurons.

As we demonstrated in rat 1a cells, the adenovirus-mediated expression of ΔFosB, and to a lesser extent FosB, in rat embryonic cortical cells resulted in the selective proliferation of nestin-positive cells even after the withdrawal of B27 trophic support, which promotes the survival of primary neurons *in vitro*. The surviving cells, in the presence of ΔFosB or FosB, underwent DNA replication and mostly expressed nestin, while less than half of them expressed GFAP also. Some of the cells expressing ΔFosB or FosB, which dominantly expressed nestin over GFAP, were morphologically most likely radial glial cells, while those with dominant expression of GFAP were most likely astrocytes (Figure 9). The decline in ΔFosB expression in these cells converted some of them to MAP2-positive, thus suggesting that ΔFosB promotes the proliferation of neuronal precursor cells without their neuronal maturation, at least *in vitro*.

The injection of recombinant adenovirus into the ventricle of the rat brain revealed that the expression of ΔFosB in cells within the ventricle wall indeed increased nestin immunoreactivity, but not GFAP immunoreactivity, as we observed in both the rat brain after ischemic insult and adenovirus-infected cortical cell culture, thus indicating that ΔFosB may stimulate proliferation of neuronal precursor cells, and as a result, the expression of nestin is likely to be upregulated. It has recently been shown that two distinct subpopulations (type I and type II) of nestin-positive cells are present in the adult mouse DG.[31] Type I cells have a lower input resistance

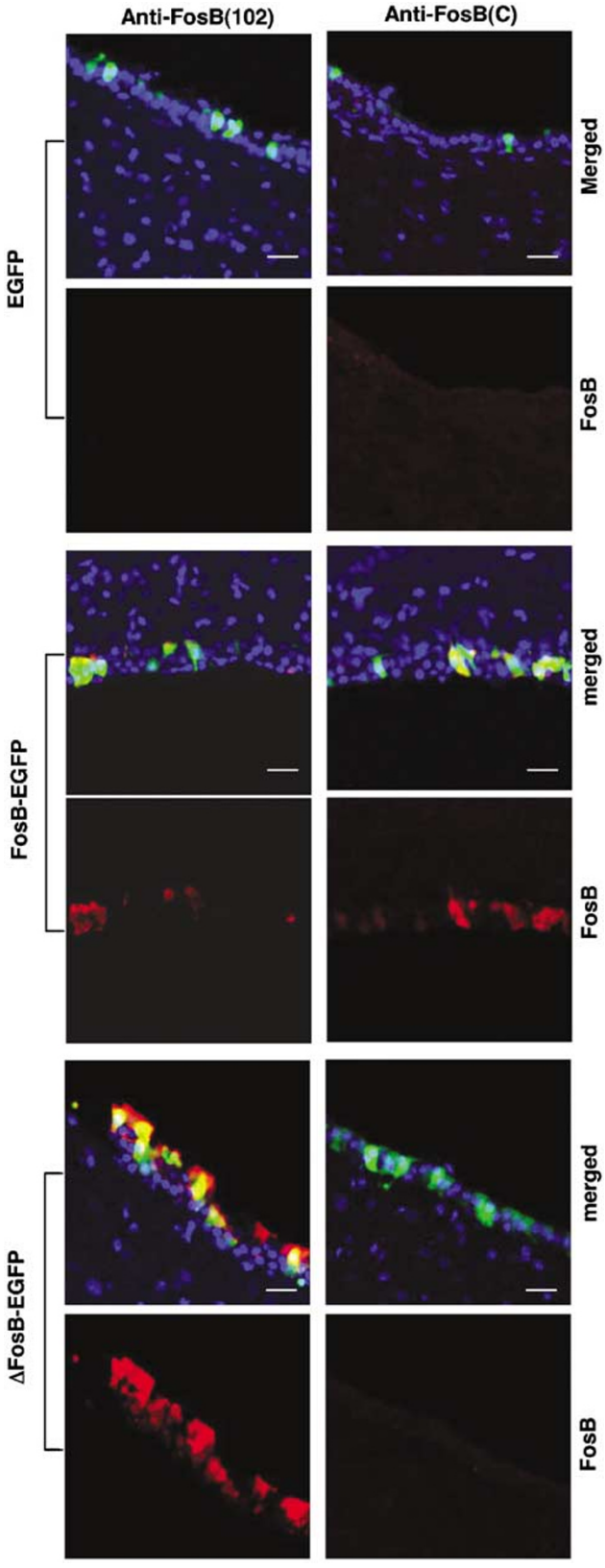


**Figure 11** Adenovirus-mediated expression of FosB and ΔFosB in cells within the ventricle wall of the rat brain. The brain sections were prepared from the rats 54 h after the adenovirus injection, and then were subjected to confocal laser-scanning microscopy after reaction with anti-FosB(102) or anti-FosB(C) with Alexa-labeled second antibody (red). The nuclei were counterstained with DAPI (blue). Infected cells were identified by EGFP fluorescence (green). Merged: the merged images. In the sections from the brain infected with adenovirus encoding EGFP alone (EGFP) or ΔFosB-EGFP (ΔFosB-EGFP), EGFP-positive cells were mostly detected in the third ventricle wall, while in the sections from the brain infected with adenovirus encoding FosB-EGFP (FosB-EGFP), EGFP-positive cells were mostly detected in the LV wall. Scale bars: 25 $\mu$m

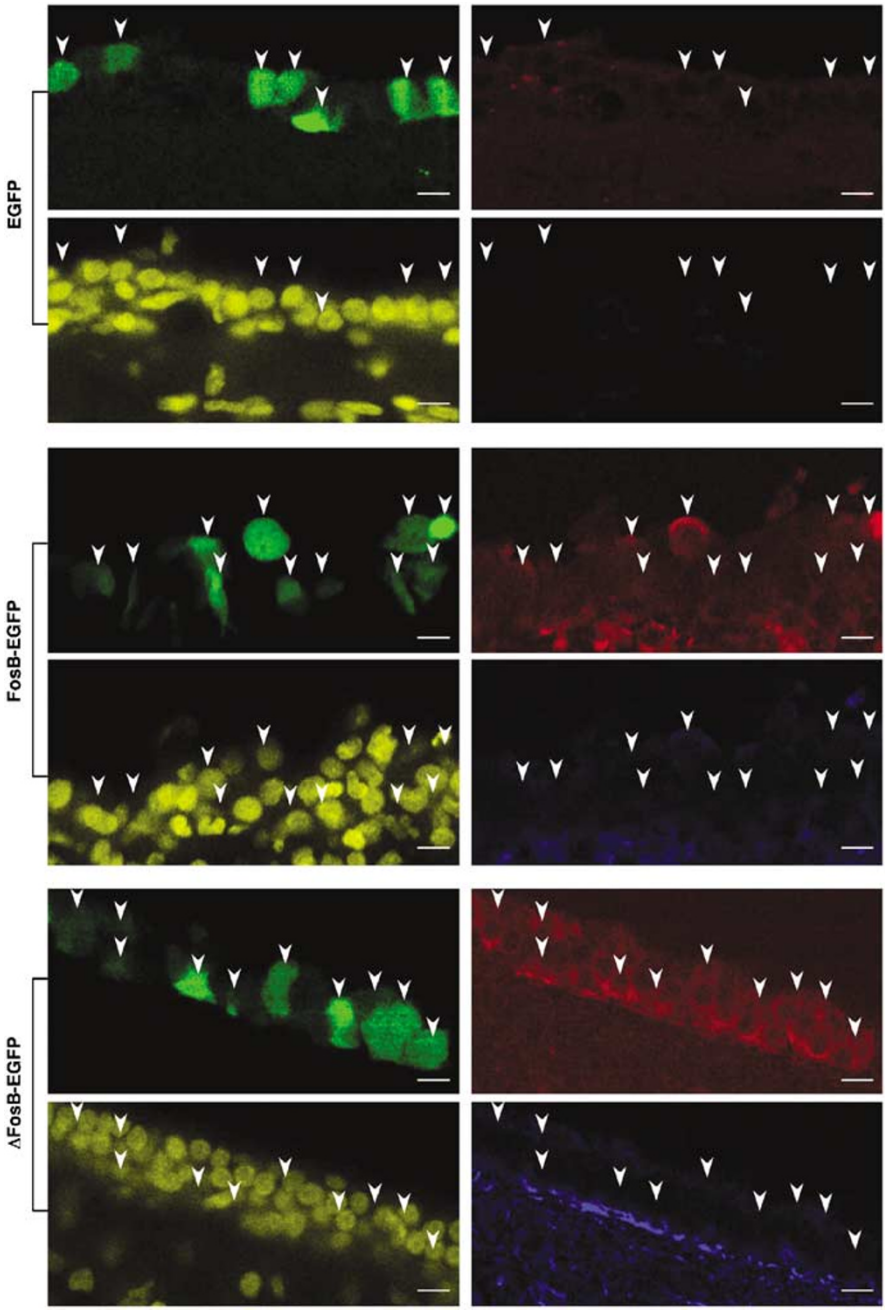


**Figure 12** ΔFosB expression in cells within the ventricle wall induces nestin expression. The brain sections were prepared as described in Figure 11, and subjected to laser-scaning confocal microscopy after reaction with anti-nestin (red) and anti-GFAP (blue) with proper Alexa-labeled second antibodies, and infected cells (arrowheads) were identified by EGFP fluorescence (green). The nuclei were counterstained with DAPI (yellow). In the sections from the brain infected with adenovirus encoding EGFP alone (EGFP), both nestin and GFAP were barely detectable. In the brain sections infected with adenovirus encoding ΔFosB-EGFP (ΔFosB-EGFP) or FosB-EGFP (FosB-EGFP), nestin immunoreactivity mostly increased in the EGFP-positive cells, while an increased GFAP immunoreactivity was seen in an adjacent layer to the EGFP-positive cell layer. Scale bars: 10 $\mu$m

value, and their radial processes are GFAP-positive, whereas type II cells have a higher input resistance value and are GFAP-negative. It is suggested that there is a rapid and dynamic cell conversion of nestin-positive progenitors, from type I to type II, at an early stage of adult neurogenesis. It has recently been reported that GFAP-expressing progenitors are a principal source of constitutive neurogenesis in adult rodent forebrain,[32] thus suggesting that ΔFosB may promote the proliferation of a subset of progenitor cells, such as type II nestin-positive/GFAP-negative cells.

At 7 days after transient forebrain ischemia, the levels of FosB or ΔFosB expression in the brain returned to almost basal levels, and many newly generated cells most likely migrated to damaged places (Figure 3). It was shown that

newly generated neurons appear to replace the damaged neurons several weeks after the initial insult; thus, the decline in ΔFosB expression may be a prerequisite for such migration and/or maturation of newly generated neurons. Adenovirus-mediated expression of ΔFosB in cells within the ventricle wall resulted in an elevated expression of nestin, but not their proliferation or migration, thus suggesting that other factors that can be induced in the brain due to some stimulation or stress might be required for their proliferation and migration.

The adenovirus-mediated expression of FosB or ΔFosB resulted in their preferential localization in the cytoplasm of both rat 1a and embryonic cortical cells, thus indicating that a large part of overexpressed FosB or ΔFosB remained in the cytoplasm. The expression of FosB or ΔFosB in the embryonic cortical cells resulted in an increased expression of each Jun protein, and JunD protein was preferentially detected in the nuclei among them (Figure 5). These results suggest that a part of overexpressed FosB or ΔFosB formed functional AP-1 heterodimers with JunD and to a lesser extent with c-Jun or JunB, as we previously demonstrated in the rat 1a embryonic fibroblasts.[18] However, we could not rule out the possibility that cytoplasmic FosB or ΔFosB may play some role in these cells.

Semiquantitative RT-PCR analyses showed that the expression of *Mmp3* gene, which is known to be upregulated by FosB but not ΔFosB in rat 1a cells as an AP-1-responsive gene,[18] was not detected in any of the embryonic cortical cells even with the expression of FosB. Furthermore, the expression of *Cdk5* and *GluR2* genes, which are known to be upregulated in the striatal neurons of ΔFosB-transgenic mice, was not altered in any of the embryonic cortical cells regardless of the FosB or ΔFosB expression, thus suggesting that in the embryonic cortical cells, neither FosB nor ΔFosB can alter the expression of known targets of FosB or ΔFosB in fibroblasts or neurons. However, we found that the expression of the *JunB* gene among the three *Jun* genes increased most remarkably in the embryonic cortical cells expressing FosB but not ΔFosB, thus suggesting that FosB or ΔFosB is involved in the regulation of a different set of genes in the embryonic cortical cells in comparison to those in fibroblasts or neurons. A functional analysis of FosB or ΔFosB on *JunB* expression may shed light on the mechanism of how FosB or ΔFosB regulates gene expression in the neuronal precursor cells.

Semiquantitative RT-PCR analyses also revealed that the increased expression of nestin and GFAP in the embryonic cortical cells by FosB or ΔFosB is partly due to the increased levels of their transcripts. It has been shown that the ectopic expression of ΔFosB in transgenic mice altered the gene expression profiles in neurons, and some were upregulated while others were downregulated.[10,11] Our data suggest that ΔFosB lacking the C-terminal transactivating domain in FosB can upregulate the expression of *Nestin* and *Gfap* genes in neuronal precursor cells, thus promoting their proliferation.

We recently identified galectin-1 to be a secretory factor whose expression is induced by ΔFosB in embryonic cell lines,[20–22] and we herein showed the expression of galectin-1 to be significantly upregulated in the embryonic cortical cells by either FosB or ΔFosB, as well as in the hippocampus after ischemia. Furthermore, our data also indicate that galectin-1, as a secretory factor from FosB- or ΔFosB-expressing cells, appears to promote the proliferation of embryonic cortical cells. We are now examining whether galectin-1 participates in such a proliferative response in the damaged brain.

It has been considered that efficient propagation of neuronal precursor cells *in vitro* is one of the most critical factors for stem cell therapy for neurodegenerative disorders. Our results strongly suggest that ΔFosB efficiently promotes the selective proliferation of neuronal precursors without their maturation even under a certain stressed condition, thus providing us with clues to develop new approaches to stem cell therapy.

## Materials and Methods

### Antibodies

The FosB(C) antibody was raised against amino acids 245–315 of the C-terminus of FosB.[5] Since the C-terminus is missing in ΔFosB, the FosB(C) antibody only recognizes FosB.[18] Rabbit polyclonal antibodies against c-Jun and JunB have been described previously.[33,34] For preparation of anti-JunD antibody, TrpE-JunD (1–92 aa) fusion protein was immunized to rabbits and antiserum was affinity purified as described previously.[5] The FosB(102) antibody (sc-48, sc-48G), and rabbit or goat polyclonal antibodies whose epitope was mapped within a common central domain (residues 75–150) of FosB and ΔFosB were products of Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibodies to glial fibrillary acidic protein (GFAP) were purchased from DakoCytomation (Kyoto, Japan). Mouse monoclonal antibodies to GFAP (G-A-5), $\beta$-tubulin III (SDL.3D10) and MAP2 (HM-2) were products of Sigma (St. Louis, MO, USA). Mouse monoclonal antibody to nestin (Rat401) was obtained from BD Bioscience Pharmingen (San Diego, CA, USA), and mouse monoclonal antibody to BrdU (BMC9318) was obtained from Roche Diagnostics Japan (Tokyo, Japan). Alexa-labeled second antibodies were obtained from Invitrogen Japan (Tokyo, Japan). The rabbit polyclonal antibodies (anti-rhGal-1) against recombinant human galectin-1 have been described previously.[35]

### Transient forebrain ischemia model

All animal experiments were conducted in accordance with the national prescribed guidelines, and ethical approval for the present studies was granted by the Animal Experiment Committee of Kyushu University. Transient forebrain ischemia was performed using published modifications[23] of the 2-VO method.[36] Briefly, male stroke-prone spontaneously hypertensive rats (SHRSP/izm, Japan SLC, Hamamatsu, Japan) weighing 250–300 g and aged 13–15 weeks were anesthetized with halothane. Both common carotid arteries were exposed and separated, and then were loosely encircled with polyethylene tubes for later ligation. Forebrain ischemia was achieved by tightening polyethylene tubes for 20 min. The brain temperature, measured indirectly via a thermocouple probe placed in the temporalis muscle, was maintained at close to 37°C throughout the operations. To detect dividing cells in the brain, BrdU (50 mg/kg; Sigma) was given to rats by intraperitoneal injection once a day after the operation for 7 days.

### Immunohistochemistry

Rats were deeply anesthetized with 1.5%. halothane, and perfused transcardially with 50 ml of heparinized saline (0.9%) followed by 150 ml of 0.1 M PBS containing 4% paraformaldehyde. The brains were fixed in 4% paraformaldehyde at 4°C for 12–24 h and embedded in paraffin. Coronal

sections (4 $\mu$m) were deparaffinized, pretreated in 3% hydrogen peroxide in methanol and subjected to immunohistochemistry with each antibody. Sections were processed using the Vectastain ABC or ABC-AP KITs (Vector laboratories, Burlingame, CA, USA) with a proper biotinylated secondary antibody, and peroxidase reaction product was detected using 3′3′-diaminobenzidine-tetrahydrochloride (Sigma), and alkaline phosphatase reaction product was detected with Vector Blue (Vector Laboratories). Digital images were acquired using Axioskop2 plus equipped with AxioCam (Carl Zeiss Japan, Tokyo).

## Rat embryonic cortical cell primary culture

Cultures of embryonic cortical cells were prepared from embryonic day 18 Wister Kyoto (WKY) rats (Kyudo, Kumamoto, Japan), as described.[26] Cells were plated at an indicated density on polyethylenimine (PEI) (Sigma)-coated glass coverslips or 96-well plates in Neurobasal medium with B27 supplement (Invitrogen) containing penicillin/streptomycin (100 U/ml), 0.5 mM L-glutamine and 10 $\mu$M 2-mercaptoethanol. Cultures were maintained in a 95% room air and 5% $CO_2$ humidified atmosphere at 37°C. Rat embryonic cortical cells cultured on PEI-coated glass coverslips were fixed in 0.1 M PBS containing 4% paraformaldehyde for 15 min, and were processed for immunofluorescence microscopy.

## Galectin-1 preparation

The recombinant mouse galectin-1$\alpha$ and galectin-1$\beta$ were prepared as described previously.[22]

## Adenovirus vector

Adenovirus vector expressing FosB or ΔFosB was constructed using Adeno-X Tet-Off Expression System 1, according to the user manual (PT3496-1, BD Biosciences Clontech, Palo Alto, CA, USA). Briefly, an *Eco*RI–*Bam*HI fragment containing the entire coding region of mouse FosB or ΔFosB was subcloned into pIRES2-EGFP vector (Clontech). An *Nhe*I–*Not*I fragment containing the entire coding region of mouse FosB or ΔFosB and an IRES followed by EGFP coding region was inserted into the *Xba*I–*Not*I sites of the pTRE-Shuttle vector. A fragment containing FosB or ΔFosB with EGFP placed under the control of Tet-responsive expression cassette was excised by I-*Ceu*I and PI-*Sce*I, and inserted into Adeno-X system 1 viral DNA, which is a derivative of a replication-incompetent (ΔE1/ΔE3) human adenoviral type 5 genome. *Pac*I-linearized recombinant Adeno-X viral DNA was transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen, Japan) according to the manufacturer's instruction manual. Harvested recombinant adenovirus was propagated and titrated on HEK293 cells. The recombinant adenoviruses obtained were purified by Adeno-X Virus Purification Kits (Clontech). The titer of each viral stock was $\sim 1.85 \times 10^9$ PFU/ml for Adeno-X:TRE-FosB-EGFP (FosB-EGFP), $\sim 2.62 \times 10^9$ PFU/ml for Adeno-X: TRE-ΔFosB-EGFP (ΔFosB-EGFP), $\sim 7.79 \times 10^8$ PFU/ml for Adeno-X:TRE-EGFP (EGFP alone) and $\sim 1.85 \times 10^9$ PFU/ml for Adeno-X Tet-Off virus. Cultured cells were infected with various recombinant adenoviruses at an MOI of 1 PFU/cell. Cells were coinfected with Adeno-X Tet-Off virus to supply tTA, a tetracycline-controlled transactivator.

## Real-time RT-PCR analysis

The primers used to amplify the 5′ common region for *fosB* and *ΔfosB* mRNA (FB1775F: GAGGAAAAGGCAGAGCTGGA; FB1855R: TGGGCC ACCAGGACAAACT), the 3′ region specific for *fosB* (FBO1925F: CCAGGGTCAACATCCGCT; FBO2016R: CGTCTCGGCTGCTCTGGA) and primers used to amplify the *c-Jun* mRNA (CJN807F: CTCCAAGTG CCGGAAAAGG; CJN961R: TGTTAACGTGGTTCATGACTTTCTG) were obtained from FASMAC Co., Ltd. (Kanagawa, Japan). Specific TaqMan probes labeled with FAM (5') and TAMURA (3') for real-time PCR detection (FB1797T: CGGAGATCGCCGAGCTGCAAAA; FBO1965T: TGCTGCCGCCCCCTCCA; and CJN905T: ACATGCTCAGGGAACAGG TGGCACAG were obtained from Applied Biosystems (Foster City, CA, USA). TaqMan gene expression assays for *JunB* (Rn0059045), *JunD* (Rn00824678), *Cdk5* (Rn00590045), *GluR2* (Rn00568514), *Mmp3* (Rn00591740), *Gfap* (Rn00566603) and *Nestin* (Rn00564394) were purchased from Applied Biosystems. Total RNA was prepared from cortical cells using RNeasy Mini kit (Qiagen KK, Tokyo, Japan), and purified RNA was treated with RNase-free DNase, according to the manufacturer's instructions. cDNAs were synthesized by first-strand cDNA synthesis kit using random hexamer as the primer (Amersham Biosciences KK, Tokyo, Japan). RT-PCR and the detection of the PCR product in real time were performed using ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Serially diluted cDNA was used to obtain a standard curve for each transcript.

## Evaluation of cell viability

Rat embryonic cortical cells were plated in 96-well PEI-coated plates at a density of $2.67 \times 10^5/cm^2$ with 10 $\mu$M AraC (Wako Pure Chemical Industries, Osaka, Japan), or $1.67 \times 10^5/cm^2$ without AraC. After withdrawal of B27 supplement from the cultures, the cell viability was measured by monosodium salt WST-8 using the Cell Counting Kit-8 (Wako). Absorbance of WST-8 formazan dye at 450 nm was measured and the relative cell viability was determined by comparing the ratio of absorbance for each experiment with that for the control experiments. In order to evaluate the viabilities of cells infected with various recombinant adenoviruses, numbers of EGFP-positive cells in 96-well plates were counted on images captured by a digital camera. The same areas were repeatedly monitored for a week, and relative cell viability was determined by comparing the ratio of cell number for each experiment to that for the control experiments.

## Decline of FosB and ΔFosB expression by Tet-off system

Rat embryonic cortical cells were cultured for 3 days in a Neurobasal medium with B27 supplement and then infected with each recombinant adenovirus together with Adeno-X Tet-Off vector. At 2 days after infection, the culture medium was changed to a Neurobasal medium lacking B27 supplement. At 13 days after infection, the culture medium was changed to a Neurobasal medium with B27 supplement and doxycycline (final 400 ng/ml; Wako). At 17 days after infection, the cells were fixed and subjected to immunofluorescence microscopy.

## Injection of adenovirus vector

Rats were anesthetized with amobarbital (100 mg/kg i.p.), and a small burr hole was made in the parietal region (1.0 mm anterior and 1.6 mm right lateral from the bregma) with a dental drill. A 27-G needle on a Hamilton syringe was stereotaxically inserted into the right LV (3.8 mm in depth), and 6 $\mu$l of viral suspension ($\sim 2.34 \times 10^6$ PFU of each recombinant adenovirus expressing FosB-EGFP, ΔFosB-EGFP or EGFP alone, together with $\sim 5.55 \times 10^6$ PFU of Adeno-X Tet-Off virus) was injected

for over 6 min. At 54 h after injection, the rats were anesthetized and perfused transcardially, and the brains were processed as described in the Immunohistochemistry section. After cryoprotection with 25% sucrose in 0.1 M PBS, coronal sections (20 $\mu$m) were prepared and subjected to immunofluorescence microscopy.

### Laser-scanning fluorescence microscopy

Confocal images were acquired under Eclipse TE300 (Nikon, Kanagawa, Japan) equipped with the Radiance 2100 laser-scanning fluorescence microscope system (Bio-Rad Laboratories, Hercules, CA, USA).

### Image processing

All digitized images were processed for publication using the Adobe Photoshop 5.5J software package (Adobe Systems).

### Statistical analysis

The data are expressed as the mean $\pm$ S.D. All data were compared using the Mann–Whitney *U*-test. Statistical significance was accepted at a level of $P < 0.05$.

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