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Galectin-1 regulates neurogenesis in the subventricular zone and promotes functional recovery after stroke

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Abstract

Galectin-1 (Gal-1) has recently been identified as a key molecule that plays important roles in the regulation of neural progenitor cell proliferation in two neurogenic regions: the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone of the hippocampal dentate gyrus. To test the hypothesis that Gal-1 contributes to adult neurogenesis after focal ischemia, we studied the temporal profile of endogenous Gal-1 expression and the effects of human recombinant Gal-1 on neurogenesis and neurological functions in an experimental focal ischemic model. In the normal brain, Gal-1 expression was observed only in the SVZ. In the ischemic brain, Gal-1 expression was markedly upregulated in the SVZ and the area of selective neuronal death around the infarct in the striatum. The temporal profile of Gal-1 expression was correlated with that of neural progenitor cell proliferation in the SVZ of the ischemic hemisphere. Double-labeling studies revealed that Gal-1 was localized predominantly in both reactive astrocytes and SVZ astrocytes. Administration of Gal-1, which is known to have carbohydrate-binding ability, into the lateral ventricle increased neurogenesis in the ipsilateral SVZ and improved sensorimotor dysfunction after focal ischemia. By contrast, blockade of Gal-1 in the SVZ by the administration of anti-Gal-1 neutralizing antibody strongly inhibited neurogenesis and diminished neurological function. These results suggest that Gal-1 is one of the principal regulators of adult SVZ neurogenesis through its carbohydrate-binding ability and provide evidence that Gal-1 protein has a role in the improvement of sensorimotor function after stroke.

Keywords: Stroke, experimental; Behavior, animal; Subventricular zone; Neural progenitor cell; Neurogenesis; Galectin-1; Regeneration; Carbohydrate

Introduction

Endogenous neural stem/progenitor cells (NSPCs) proliferate markedly after brain injury, including stroke, in the adult mammalian brain (Arvidsson et al., 2002; Parent et al., 2002; Tonchev et al., 2003a,b). These cells persist mainly in the subventricular zone (SVZ) along the lateral walls of the lateral ventricles (LVs) and in the subgranular layer (SGL) of the dentate gyrus (DG) in the adult mammalian forebrain (Lois and Alvarez-Buylla, 1993; Luskin, 1993; Eriksson et al., 1998). Newly generated NSPCs migrate to the site of injury as neuroblasts and some differentiate into mature neurons around the injured area by the process of neurogenesis (Arvidsson et al., 2002; Parent et al., 2002; Yamashita et al., 2006). Because neurogenesis may be linked to functional recovery after stroke (Nakatomi et al., 2002; Zhang et al., 2002; Raber et al., 2004), understanding the mechanisms of NSPC proliferation after brain injury is the first important step in developing neuro-regenerative strategies. Although various brain injuries have been reported to promote NSPC proliferation in the SVZ and SGL (Cameron et al., 1998), their regulatory role after brain injury is largely unknown.

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Galectin-1 (Gal-1) is the first identified member of the galectin family of carbohydrate-binding proteins that has been implicated in a variety of biological events (Barondes et al., 1994). Gal-1 exists in either a reduced or oxidized form, and only the reduced form possesses carbohydrate-binding activity (Whitney et al., 1986). The different functional roles of oxidized or reduced Gal-1 are known in the nervous system (Camby et al., 2006). The oxidized Gal-1 has been shown to promote neurite outgrowth in peripheral (Inagaki et al., 2000; Horie et al., 2004) and central (McGraw et al., 2005) nerves. Furthermore, the neuroprotective effect of oxidized Gal-1 has also been shown in a transgenic murine amyotrophic lateral sclerosis (ALS) model (Chang-Hong et al., 2005). However, these effects on the nervous system are observed with the oxidized form of Gal-1, which is known to lose carbohydrate-binding activity.

In our recent report, reduced Gal-1 was identified as a key molecule in the regulation of adult endogenous NSPC proliferation *in vivo* and neurosphere formation *in vitro* (Sakaguchi et al., 2006). The current understanding of adult neurogenesis suggests that neural stem cells (NSCs) in the adult SVZ express glial fibrillary acidic protein (GFAP) and exhibit structural characteristics of astroglia (Doetsch et al., 1999); these multipotent SVZ astrocytes, in turn, give rise to new neurons (Doetsch et al., 1999). In the normal adult mouse brain, endogenous Gal-1 is expressed in these SVZ GFAP-expressing astrocytes and regulates the proliferation of NSPCs in the SVZ through its carbohydrate-binding ability (Sakaguchi et al., 2006). However, the effect of Gal-1 on the proliferation of NSPCs after brain injury was not studied. This endogenous protein is potentially involved in ischemia-driven neurogenesis and thus is potentially applicable as a new drug in regenerative therapy of stroke.

To investigate the endogenous Gal-1 expression sites after stroke, we used an experimental focal ischemic rodent model to quantify the time profile of expression of Gal-1 and a cell proliferation marker. Furthermore, to investigate the therapeutic potential of Gal-1 administration for stroke, we tested the infusion of both the oxidized and reduced forms of Gal-1 into the LV of the ischemic brain.

Materials and methods

Animals and experiments

Our study was conducted in accordance with NIH guidelines for the care and use of animals in research and under protocols approved by the Animal Experimental Committee of Tokyo Medical and Dental University. We minimized the number of animals used, and we used anesthesia appropriately to minimize suffering. We used male gerbils ($n=70$, Sankyo Laboratory Animal Center, Tokyo, Japan) that were 16–18 weeks of age and weighed 65–72 g.

Focal ischemic surgery

Focal ischemic surgery was conducted by our previously described method (Ohno et al., 1984; Ishibashi et al., 2003). In brief, the left common carotid artery was occluded with a mini vascular clip under anesthesia, and ischemic animals were selected according to a stroke index (Ohno et al., 1984). Total occlusion time was 20 min (two 10-min occlusions 5 h apart). These ischemic animals were randomly divided into two groups, group 1 animals ($n=25$) and group 2 animals ($n=40$). Sham-operated animals ($n=5$) were operated upon in the same manner but were not subjected to left carotid artery occlusion.

BrdU labeling

To label S-phase cells, the thymidine analog 5-bromo-2′-deoxyuridine-5′-monophosphate (BrdU) was administered intraperitoneally (50 mg/kg; Sigma, St. Louis, MO). Two injection protocols were used. In group 1 animals ($n=25$) and sham-operated animals ($n=5$), we gave two injections (6 h apart) of BrdU, 24 h before they were euthanized by transcardiac perfusion (Fig. 1A). This allowed us to measure the number of cells that incorporated BrdU during a 24-h period and provided an index of the rate of cell birth at a specific time point after ischemia. In group 2 animals ($n=40$), we gave injections of BrdU twice daily for 7 consecutive days during Gal-1 treatment period (Fig. 1B). This allowed us to investigate the phenotype, survival and migration pattern of newborn cells during and after treatment.

Intraventricular administration of Gal-1

Gal-1 loses its carbohydrate-binding ability upon being oxidized (Inagaki et al., 2000). To elucidate whether the carbohydrate-binding ability was essential for Gal-1 function in NSPC proliferation in the SVZ after ischemia, we used both oxidized-galectin-1 (Ox-Gal-1) and CS-galectin-1 (CS-Gal-1). CS (cysteine to serine)-Gal-1 retains its carbohydrate-binding activity under oxidative conditions since all the cysteines responsible for Gal-1 oxidation are converted to serine (Inagaki et al., 2000). CS-Gal-1 (Kirin, Gunma, Japan), Ox-Gal-1 (Kirin), anti-Gal-1 neutralizing antibody (Kirin, rabbit IgG) or control rabbit IgG (Sigma, St. Louis, MO) was administered intraventricularly to the non-ischemic hemisphere of group 2 animals once a day from Day 1 to Day 7 after focal ischemia (Fig. 1B).

One day after surgery, the animal was mounted and secured in a stereotaxic frame under anesthesia. A cut was made directly down the midline to expose the skull. The skull surface was cleaned and dried with sterile gauze and cotton tips, and a 24-gauge guide cannula, 8 mm length was implanted following the coordinates given in the atlas (Loskota et al., 1974). This point was 0.5 mm posterior to the bregma (AP), 1.4 mm lateral (right) to the midline (L) and 2.0 mm deep from the level of the lateral (DV). The guide cannula was then solidly fixed with dental cement.

The animals received an intrathecal (IT) injection of anti-Gal-1 antibody (30 μg/ml), Ox-Gal-1 (10 μg/ml), CS-Gal-1 (10 μg/ml) or rabbit IgG antibody (30 μg/ml) once a day for 7 days. A total volume of 8 μl of drug solution was infused with a micro-pump injector, through a 28-gauge internal cannula

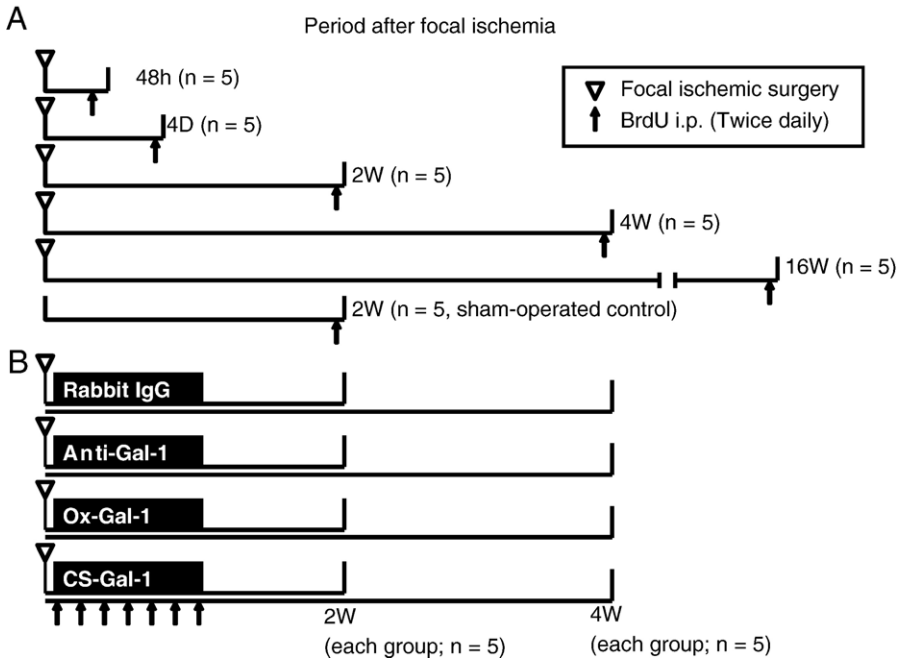


Fig. 1. Experimental protocol used for BrdU and galectin-1 administration.

(Unique Medical, Tokyo, Japan) over 2 min, and the injector was left in place for a further 2 min before removal. The animals were then returned to their home cages.

Tissue preparation and measurement of total infarction volume

At the end of the observation period, the animals were deeply anesthetized with diethylether and fixed by transcardiac perfusion with 4% paraformaldehyde. The brains of group 1 animals were embedded in paraffin, and the brains of group 2 animals were incubated in 25% sucrose for 48 h, frozen rapidly in dry ice. The brains were then cut into 6-μm or 10-μm coronal sections, mounted on the slides and processed for staining. To evaluate the ischemic lesions, 6-μm coronal sections at bregma levels +2.10, +1.00, −0.20, −1.20, −2.30 and −3.30 mm (Loskota et al., 1974) were stained with K.B (Klüver-Barrera). Selective neuronal death was defined as scattered neurons with pyknosis and shrunken cytoplasm. Infarction was defined as an area of tissue within which all cells, blood vessels and the neuropil were destroyed. The areas of the infarction, the ipsilateral hemisphere and the contralateral hemisphere in the 6 coronal sections were measured on an image of each section by using the National Institutes of Health Image analysis software (version 1.55). The total infarction volume of the ipsilateral hemisphere (% infarction volume) was calculated as a percentage of the volume of the contralateral hemisphere, as previously reported (Swanson et al., 1990).

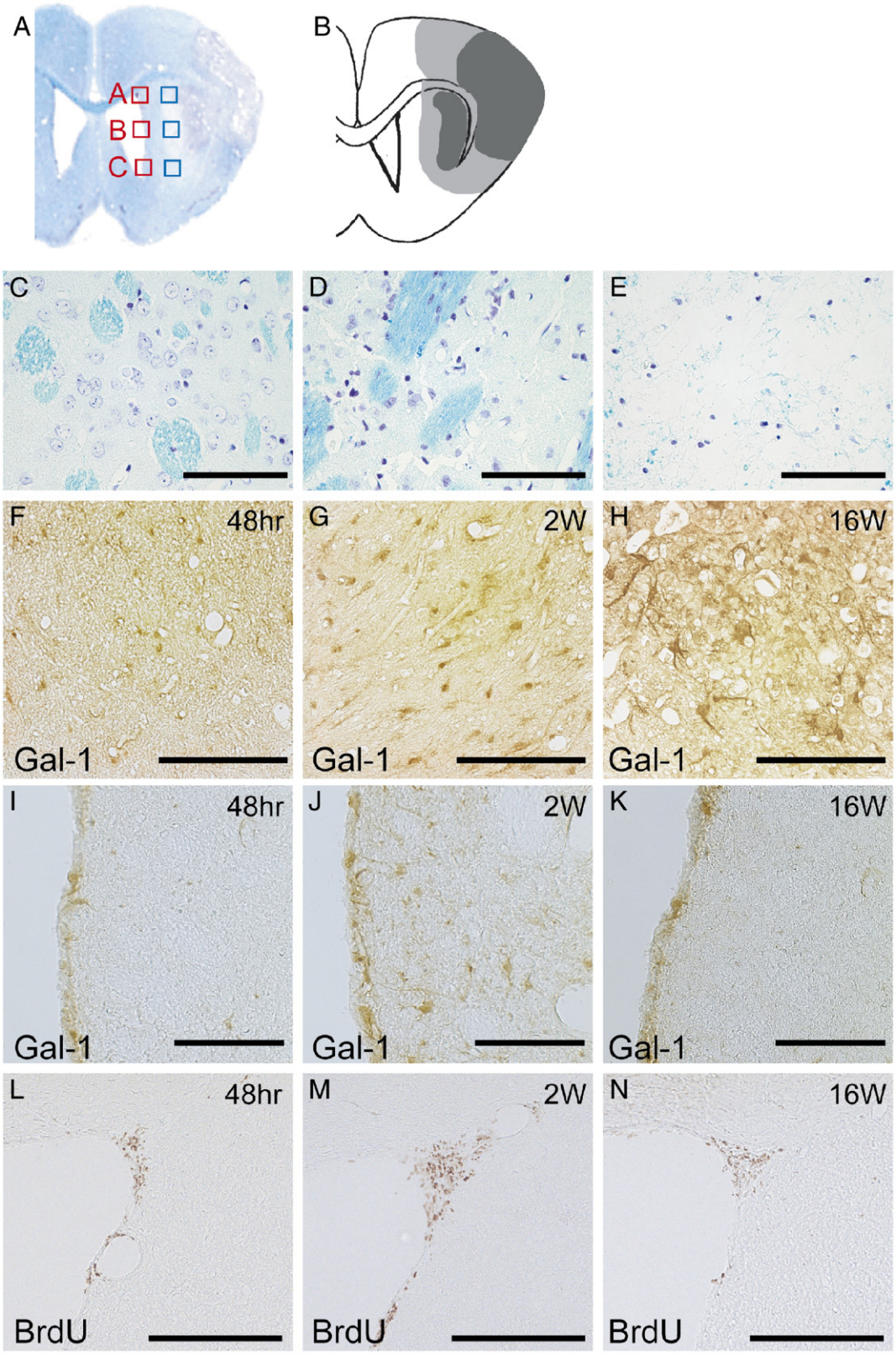
Immunohistochemistry

Immunostaining of Gal-1-immunoreactive cells in the cerebral hemisphere was performed with a rabbit polyclonal anti-Gal-1 antibody (Kirin). The 6-μm coronal sections were rinsed in PBS, preincubated in PBS with 10% normal goat serum for 60 min at room temperature and incubated overnight at 4 °C in rabbit anti-Gal-1 antibody, 1:500. Immunostaining of BrdU required DNA denaturing. The sections were incubated in 2 N HCl at 37 °C for 30 min, rinsed in PBS, preincubated in PBS with 10% normal goat serum for 60 min at room temperature and incubated overnight at 4 °C in rat anti-BrdU antibody (Chemicon, Temecula, CA), 1:500. Subsequently, the avidin–biotin–peroxidase complex method was applied and the peroxidase reaction was developed with diaminobenzidine.

Double immunostaining was performed by immunofluorescence. For double-labeling of Gal-1 or BrdU and cell-specific markers, 10-μm coronal sections were incubated with the following antibodies: rabbit anti-Gal-1, 1:500; rat anti-BrdU, 1:500; mouse anti-neuronal nuclei antibody (NeuN for neurons), 1:500 (Chemicon); mouse anti-glial fibrillary acidic

Fig. 2. Gal-1 expression and the cell proliferation marker BrdU are upregulated after focal ischemia. Photographic display (A), schematic drawing (B) and microscopical pictures (C-E) of representative K.B. (Klüver-Barrera)-stained coronal brain sections taken from an ischemic animal. (A) Six regions of interest (ROIs, 0.3 × 0.3 mm) were defined within the ipsilateral side of SVZ (red square) and striatum (blue square). (B) Infarct (dark gray) in the cerebral cortex and striatum surrounded by widespread selective neuronal death (light gray) were detected in ischemic animals. Normal striatum from sham-operated animal (C). Reduced number of surviving neurons in the area of selective neuronal death (D). No surviving neurons in the area of infarction (E). Immunohistochemical coronal sections of the striatum (F-H) and SVZ (I-N) of the ipsilateral hemisphere showed increased number of Gal-1-immunoreactive cells and BrdU-immunoreactive cells after focal ischemia. Gal-1-immunoreactive cells were detected in the ischemic striatum 48 h (F) after induction of focal ischemia, had increased markedly in number by 2 weeks (G) and persisted until 16 weeks (H) after focal ischemia induction.Gal-1-immunoreactive cells in the ipsilateral SVZ were present 48 h (I) after induction of focal ischemia, had transiently increased in number by 2 weeks (J) and had decreased in number by 16 weeks (K). BrdU-immunoreactive cells in the SVZ were present 48 h (L) after induction of focal ischemia, had transiently increased in number by 2 weeks (M) and had decreased in number by 16 weeks (N). *Bars = 100 μm (C-K); 200 μm (L-N).*

protein (GFAP for astrocytes), 1:500 (Chemicon); mouse IgM anti-PSA-NCAM (PSA-NCAM for neuroblasts), 1:500 (a kind gift from Tatsunori Seki) (Seki, 2002); and rabbit anti-Iba1 for microglia, 1:500 (Wako, Tokyo, Japan). Subsequently, sections were incubated in fluorescent-labeled secondary antibodies (FITC/Rhodamine, raised in goat; Chemicon) and observed under a confocal laser scanning microscope (Zeiss, LSM510).



Cell counting in the SVZ and striatum

To determine the number of BrdU-labeled or Gal-1-labeled cells, six slide-mounted coronal sections, 200 μm apart, between bregma levels +1.1 and −0.2 mm (Loskota et al., 1974) were sampled for each brain. In each coronal section, 3 regions of interest (ROIs) were determined at the top, the middle and the bottom of the SVZ along the LV and 3 ROIs in the striatum were determined 1.0 mm lateral from the SVZ ROIs. These striatal ROIs were located in the area of selective neuronal death around the striatal infarction, as described in our previous study (Sun et al., 2006). For example, at the level of +0.7 mm from bregma, as shown in Fig. 2A (a representative coronal section), 3 SVZ ROIs were set at the following, lateral and depth from bregma coordinates; ROI A 0.8 mm, 2.0 mm; ROI B 0.8 mm, 2.6 mm; ROI C 0.8 mm, 3.2 mm, according to the atlas (Loskota et al., 1974). To estimate the migration of BrdU-labeled postmitotic cells from the SVZ to the olfactory bulb (OB), three slide-mounted sections, 200 μm apart, between bregma level +3.0 and +2.1 mm were selected. In each coronal section, 3 ROIs were situated in the cellular layers of the ipsilateral OB.

To identify the phenotypes of BrdU-labeled cells, six coronal sections, 200 μm apart, between bregma levels +1.1 and −0.2 mm were selected. Double immunofluorescence-stained sections were examined with a Zeiss M510 laser scanning confocal imaging system. Cells double-labeled with BrdU and a phenotype-specific marker (PSA-NCAM or NeuN) were identified in the same ipsilateral SVZ and striatal ROIs of six sliced coronal sections as described above. Double labeling was verified by scanning the cell in its entirety within the section by focusing through the *z*-axis.

In each case, measurements were made in a predefined field (0.3 × 0.3 mm). The average of the number of cells in each 3 ROIs in the ipsilateral SVZ, striatum or OB of the three or six sliced coronal sections was calculated.

Evaluation of neurological functions

To investigate the effect of Gal-1 on neurological functions, group 2 animals were subjected to a series of behavioral tests during the 14 or 28 days after focal ischemia was induced. The researcher conducting the behavioral testing and scoring was blind to the experimental conditions. All animals were videotaped during behavioral tests.

The elevated body swing test (EBST) was used to evaluate asymmetric motor behavior (Borlongan and Sanberg, 1995). Animals were held by the base of the tail and elevated about 10 cm from the tabletop. The direction of body swing, defined as an upper body turn of >10° to either side, was recorded for 1 min during each of three trials per day. The numbers of left and right turns were counted, and the percentage of turns made to the side contralateral to the lesioned hemisphere (% right-biased swing) was determined.

The bilateral asymmetry test (BAT) is a test of unilateral sensory dysfunction (Hoyman et al., 1979). Two small pieces of adhesive-backed paper dots were used as bilateral tactile stimuli occupying the distal–radial region on the wrist of each forelimb. The time, to a maximum 3 min, that it took for each gerbil to remove each stimulus from the forelimb (removal time) was recorded in three trials per day.

The T-maze spontaneous alternation task is a method of testing spatial cognitive function (Gerlai, 1998). Animals were allowed to alternate between the left and right goal arms of a T-shaped maze throughout a 15-trial continuous alternation session. Once they had entered a particular goal arm, a door was lowered to block entry to the opposite arm. The door was re-opened only after the animals had returned to the start arm, thus allowing a new alternation trial to be started. Behavior was traced with a video-tracking system (PanLab, Barcelona, Spain). The spontaneous alternation rate was calculated as the ratio between the number of alternating choices and the total number of choices.

Statistical analyses

The results were analyzed by using one-way or repeated measures analysis of variance (ANOVA) with independent variables of treatment group and day of testing, followed by the Bonferroni post hoc test for multiple comparisons between groups. The level of statistical significance was set at $P<0.05$. All values are presented as mean ± SD.

Results

Localization of Gal-1-immunreactive cells after focal ischemia

No groups 1 and 2 animals died during the observation period. Sham-operated controls did not show any detectable pathologic changes with K.B.-staining (Fig. 2C). In all ischemic animals, infarction (Fig. 2E) was distributed in the ipsilateral cerebral cortex, lateral striatum, hippocampus and thalamus, surrounded by an area of selective neuronal death (Fig. 2D), similar to our previous report (Ishibashi et al., 2003; Sun et al., 2006).

In sham-operated animals, Gal-1-immunoreactive cells were observed only in the SVZ. In ischemic animals, Gal-1-immunoreactive cells appeared in the area of selective neuronal death around the infarct (Figs. 2F–H) and in the SVZ (Figs. 2I–K). The number of Gal-1-immunoreactive cells in the ipsilateral striatum peaked at 2 weeks (Figs. 2G and 3A), and then decreased from 4 to 16 weeks after ischemia but remained significantly higher than in the control animals (Figs. 2H and 3A). In the ipsilateral SVZ (Figs. 2I–K), the numbers of Gal-1-immunoreactive cells at 4–14 days after ischemia were significantly higher than in the sham-operated animals (Figs. 2J and 3B). The number of Gal-1-immunoreactive cells in the ipsilateral SVZ peaked at 2 weeks (Figs. 2J and 3B) and was not significantly different from that in the controls at 4 and 16 weeks (Figs. 2K and 3B) after induction of ischemia.

To confirm the time profile of stroke-induced increase in SVZ cell proliferation, we examined BrdU labeling immunohistochemistry. Focal ischemia increased the number of BrdU-immunoreactive cell nuclei in the ipsilateral SVZ compared with that in the contralateral SVZ ($P<0.01$, data not shown). From 4 days after induction of focal ischemia, the number of BrdU-immunoreactive cells in the ipsilateral SVZ was

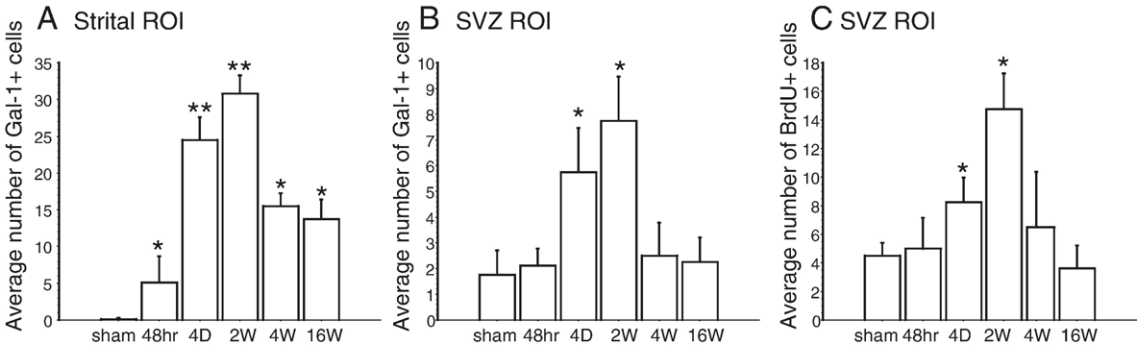


Fig. 3. Quantification of Gal-1 and BrdU-immunoreactive cells at different time points after focal ischemia induction. Average number of cells-immunoreactive for Gal-1 in the ipsilateral 3 striatal ROIs (A) and in the 3 ROIs in the ipsilateral SVZ (B) are shown, as well as average number of BrdU-immunoreactive cells in the 3 ROIs in the ipsilateral SVZ (C). *$P<0.01$; **$P<0.001$ versus sham-operated controls (one-way ANOVA with Bonferroni post hoc test).

significantly higher than that in sham-operated animals, but by 4 weeks the number was not significantly different (Figs. 2L–N and 3C). The time profile of BrdU-immunoreactive cells in the SVZ was similar to that of Gal-1-immunoreactive cells in the SVZ.

Within this time profile of Gal-1 expression, Gal-1 was found in similar cell types. A great majority of Gal-1-immunoreactive cells coexpressed GFAP both in the areas of selective neuronal death around the infarct (Fig. 4A) and in the SVZ (Fig. 4B). Almost all Gal-1-immunoreactive cells in the area of selective neuronal death showed enlargement of GFAP-positive cell bodies and processes (Fig. 4A), which is in accordance with the morphological characteristics of reactive astrocytes (Petito et al., 1990). The GFAP-immunoreactive cells were the major cell type of Gal-1-immunoreactive cells throughout the total period examined in ischemic animals, although small numbers of Gal-1-positive cells were observed among the neurons of the cerebral cortex in both sham-operated and ischemic animals (data not shown).

Infarct volume and neurogenesis in the SVZ after administration test

The total infarct volume 14 days and 28 days after focal ischemia did not differ significantly between the subgroups of group 2, at 24.5%±4.3% and 22.5%±2.5% for those given CS-Gal-1; 24.1%±5.5% and 20.8%±6.9% for Ox-Gal-1; 23.8%±4.8% and 24.2%±6.7% for anti-Gal-1; and 25.1%±5.3% and 22.2%±8.7% for rabbit IgG.

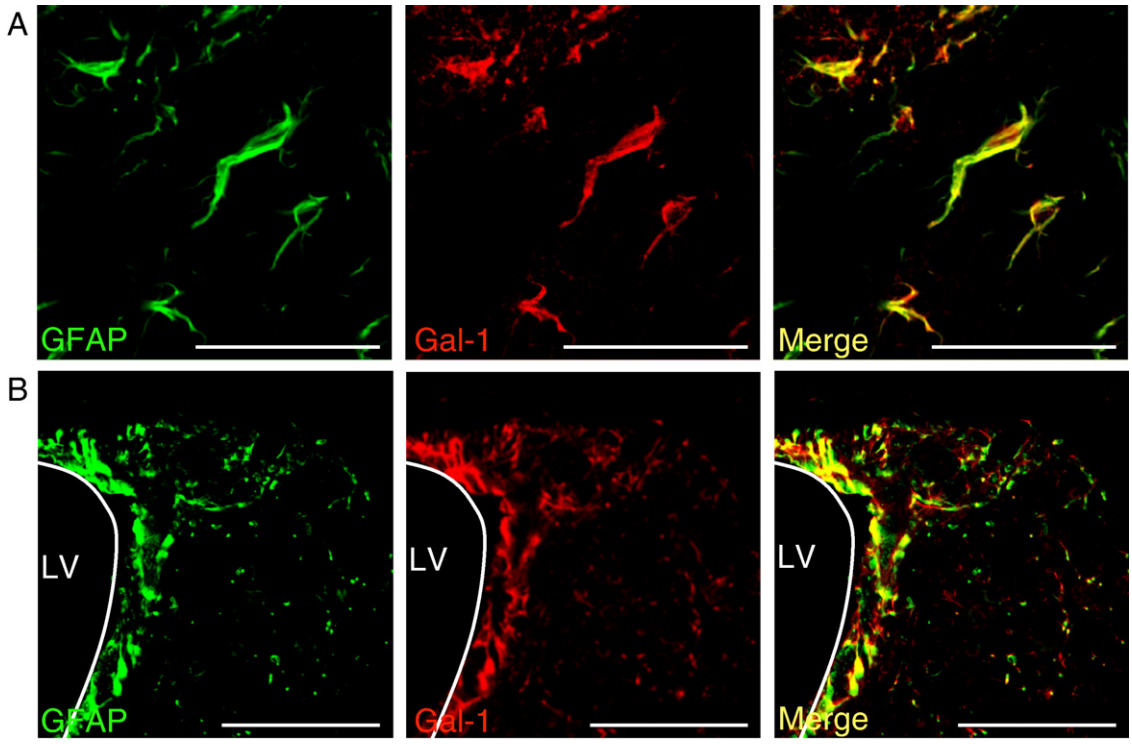


Fig. 4. Confocal immunofluorescence double labeling shows phenotypes of Gal-1-immunoreactive cells in the forebrain. Most of the Gal-1-immunoreactive cells (red) in the ipsilateral striatum (A) and SVZ (B) coexpress glial fibrillary acidic protein (GFAP; green). Scale bars=30 μm (A); 100 μm (B).

Widespread BrdU-immunoreactive cells were observed in the ipsilateral SVZ (Figs. 5A–C), striatum, cerebral cortex, hippocampus and DG 14 and 28 days after the induction of focal ischemia. The number of BrdU-immunoreactive cells in the ipsilateral SVZ was significantly increased in CS-Gal-1 animals (Fig. 5C, $P<0.01$, one-way ANOVA with Bonferroni post hoc test) and significantly decreased in anti-Gal-1 animals (Fig. 5B, $P<0.01$, one-way ANOVA with Bonferroni post hoc test), compared with that in rabbit IgG animals (controls) 14 and 28 days after focal ischemia (Fig. 5A). The number of BrdU-

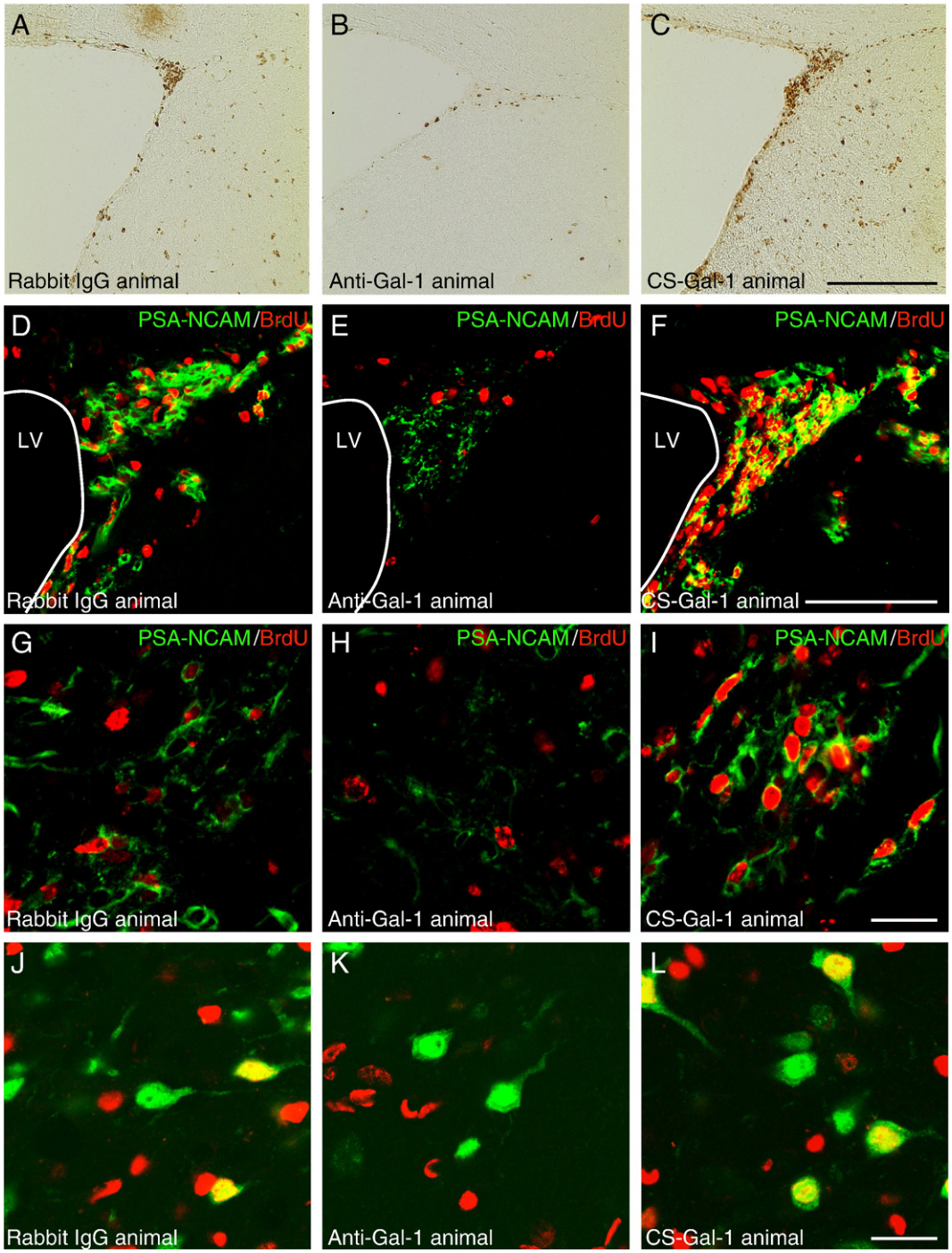


Fig. 5. Neurogenesis in the ipsilateral SVZ is enhanced by CS-Gal-1 administration. Immunohistochemical sections (A-C) of the ipsilateral SVZ 2 weeks after induction of focal ischemia show a marked increase in number of BrdU-immunoreactive cells in CS-Gal-1 animals (C) and decreased number of BrdU-immunoreactive cells in anti-Gal-1 animals (B) compared with rabbit IgG controls (A). Confocal immunofluorescence double labeling 2 weeks (D-I) and 4 weeks (J-L) after induction of focal ischemia shows the phenotypes of BrdU-immunoreactive cells in the ipsilateral SVZ and striatum. In the ipsilateral SVZ (D-F) and striatum (G-I), the number of BrdU (red)/PSA-NCAM (green) double positive cells is greater in CS-Gal-1 animals (F, I) and less in anti-Gal-1 animals (E, H), compared with rabbit IgG controls (D, G). In the ipsilateral striatum (J-L), the number of BrdU (red)/NeuN (green) double positive cells (yellow nucleus) is greater in CS-Gal-1 animals (L) and less in anti-Gal-1 animals (K) compared with rabbit IgG controls (J). NeuN stained both the cell nucleus and perinuclear cytoplasm in most of the striatal neurons. Scale bars = 200 μm (A-C); 100 μm (D-F); 20 μm (G-L).

immunoreactive cells in Ox-Gal-1 animals, however, did not change compared with that in rabbit IgG animals.

In the ischemic core, no NeuN-immunoreactive or PSA-NCAM-immunoreactive cells were observed. The majority of BrdU-immunoreactive cells in the ischemic core were identified as Iba-1-immunoreactive microglia/macrophages (data not shown). At 14 days after focal ischemia, many BrdU-immunoreactive cell nuclei were identified as PSA-NCAM-positive neuroblasts in the ipsilateral SVZ (Figs. 5D–F) and in the ipsilateral striatum around the infarct (Figs. 5G–I). Some of the BrdU-immunoreactive cell nuclei in the ipsilateral striatum around infarct were identified as the nuclei of NeuN-positive mature neurons (Figs. 5J–L). The number of PSA-NCAM and BrdU double-positive cells in the ipsilateral SVZ was lower at 28 days than that at 14 days after focal ischemia (Fig. 6A). In contrast, the number of PSA-NCAM/BrdU and NeuN/BrdU double-positive cells in the ipsilateral striatum was higher at 28 days than that at 14 days (Figs. 6B and C). The numbers of both PSA-NCAM/BrdU and NeuN/BrdU double-positive cells in the ipsilateral SVZ, striatum and OB were significantly higher in CS-Gal-1 animals (Figs. 5F, I, L and 6A–D) and significantly lower in anti-Gal-1 animals (Figs. 5E, H, K and 6A–D) than in the controls.

Behavioral tests

Elevated body swing test (Fig. 7A)

Ischemic animals show a strong tendency to turn toward the side contralateral to the lesioned hemisphere (Fig. 7). Three weeks after treatment, CS-Gal-1 animals recovered from biased body swing, and the % right-biased swing was significantly lower in these CS-Gal-1 animals than that in rabbit IgG animals.

Bilateral asymmetry test (Fig. 7B)

The time taken to remove the adhesive stimulus from the contralateral forelimb was significantly longer than that from the ipsilateral forelimb after focal ischemic surgery. CS-Gal-1 animals showed better recovery from the contralateral sensory deficit, and anti-Gal-1 animals deteriorated sensory deficit. The removal time was significantly shorter in CS-Gal-1 animals and longer in anti-Gal-1 animals at 4 weeks after induction of ischemia than in rabbit IgG animals.

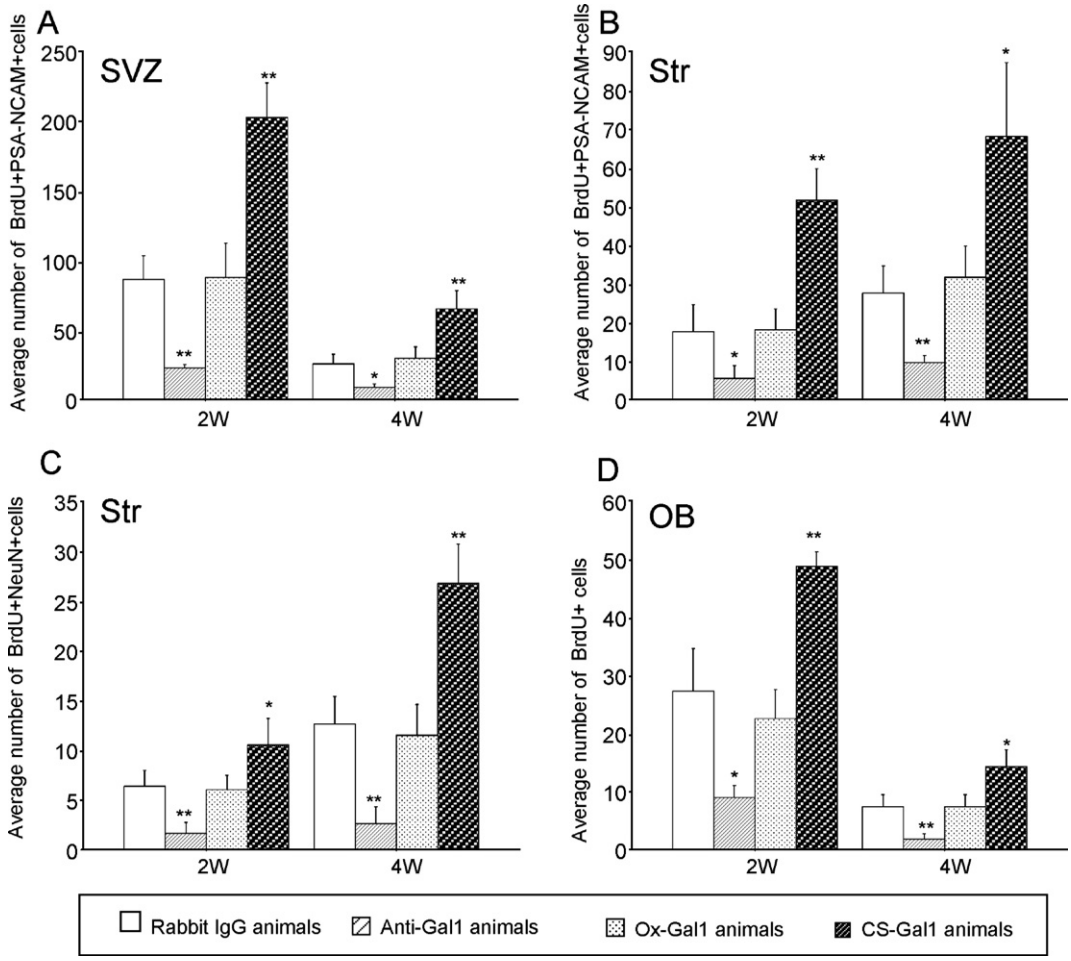


Fig. 6. Quantification of BrdU-, BrdU/PSA-NCAM- and BrdU/NeuN-immunoreactive cells after Gal-1 administration. Average number of BrdU/PSA-NCAM cells in the 3 ROIs in the ipsilateral SVZ (A), in the ipsilateral striatum (B), in the BrdU/NeuN-double positive cells in the ipsilateral 3 striatal ROIs (C) and in the BrdU-immunoreactive cells in the ipsilateral olfactory bulb (D) at 2 and 4 weeks after induction of focal ischemia are shown. SVZ, subventricular zone; Str, striatum; OB, olfactory bulb. *$P < 0.05$; **$P < 0.01$ versus rabbit IgG controls (one-way ANOVA with Bonferroni post hoc test).

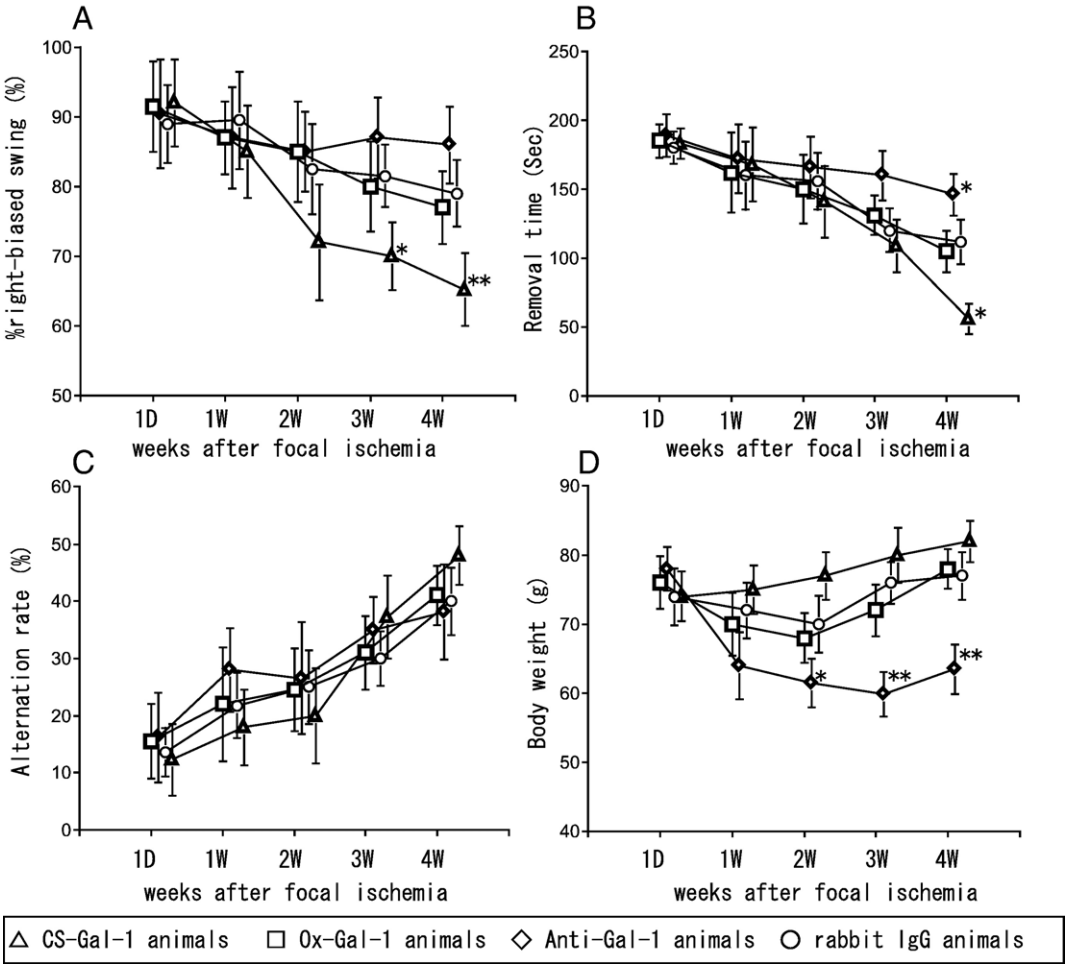


Fig. 7. Time courses of results of behavioral tests in CS-Gal-1, Ox-Gal-1, anti-Gal-1 and rabbit IgG animals over a 28-day period. (A) Percentage right-biased swing (elevated body swing test). (B) Removal time (bilateral asymmetry test). (C) Spontaneous alternation rate (T-maze test) (D) Body weight (g). Repeated-measures ANOVA showed significant effect of treatment on elevated body swing test ($P<0.01$), on removal time ($P<0.01$) and on body weight ($P<0.001$), but no significant effect on spontaneous alternation rate ($P<0.14$). *$P<0.05$; **$P<0.01$ versus rabbit IgG controls (repeated measures ANOVA with Bonferroni post hoc test; $n=5$).

T-maze test (Fig. 7C)

The T-maze test showed decreased spontaneous alternation rate in all focal ischemic animals. Four weeks postischemia, no significant difference was detected between each group.

Body weight (Fig. 7D)

Ox-Gal-1, anti-Gal-1 and rabbit IgG animals showed decreased body weight 1 and 2 weeks postischemia. In anti-Gal-1 animals, body weight was significantly decreased from 2 to 4 weeks postischemia compared with the control.

Discussion

This study demonstrates that SVZ and reactive astrocytes upregulate Gal-1 expression after focal ischemia and that Gal-1 promotes neurogenesis through its carbohydrate-binding ability. Our results also showed that Gal-1 treated animals benefited from long-term improvement in neurological function after stroke, although we are unable conclude that this functional recovery was a direct benefit of neurogenesis in these animals.

Our study utilized a gerbil model of focal ischemia to separately analyze reactive astrocytes and SVZ astrocytes, as this model results in a fully perfused area along the lateral ventricle that separates reactive astrocytes in the ischemic cerebral cortex and lateral striatum from astrocytes in the SVZ. This model has been shown previously to be adequate for this purpose, and the distribution of the ischemic lesion and the time course of neurological dysfunctions were reproducible and similar to those in our earlier reports (Ishibashi et al., 2003; Sun et al., 2006).

Gal-1 is upregulated in SVZ astrocytes and reactive astrocytes after ischemia

We found that Gal-1 expression was transiently upregulated by SVZ astrocytes in the ipsilateral hemisphere, suggesting a tight correlation between Gal-1 protein and neurogenesis after brain ischemia. The modern concept of neurogenesis in the adult brain is predicated on the premise that multipotent SVZ astrocytes give rise to new neurons (Doetsch et al., 1999). Our previous study reported that Gal-1 was expressed in SVZ astrocytes in the normal adult mouse brain and was secreted

from cultured neural stem cells *in vitro*, suggesting that Gal-1 likely functions as an autocrine or paracrine signaling mechanism in neurogenesis (Sakaguchi et al., 2006). Our present results are in accordance with these findings. In experimental focal ischemic rodent models, neurogenesis in the SVZ is reportedly activated approximately 1–2 weeks postischemia (Arvidsson et al., 2002; Parent et al., 2002). In our experiment, the number of BrdU or Gal-1-immunoreactive cells in the ipsilateral SVZ was transiently increased from 4 days to 2 weeks after ischemia.

Gal-1 expression was also upregulated in reactive astrocytes markedly proliferating around the infarct as well as SVZ astrocytes. How Gal-1 expressing reactive astrocytes in the ischemic striatum actually works is unclear at this point. It is well known that astrocytes proliferate after brain injury, and these proliferated reactive astrocytes after ischemia may be related to the proliferation of NSPCs (Ma et al., 2005). Interestingly, astrocytes from the neonatal brain increase neurogenesis from adult SVZ NSPCs (Lim and Alvarez-Buylla, 1999). In addition, astrocytes derived from the adult hippocampus promote neurogenesis from adult hippocampal NSPCs in coculture by increasing neuronal fate specification (Song et al., 2002). Furthermore, SVZ-derived neuroblasts maintain the ability to divide not only in the SVZ, but also in the ischemic striatum during migration in the focal ischemic rat (Zhang et al., 2007). Gal-1 expressing reactive astrocytes may contribute to the proliferation of NSPCs around the ischemic lesion.

Gal-1 administration promotes neurogenesis thorough its carbohydrate binding ability

Exogenous Gal-1 administration significantly increased the proliferation of cells in the ipsilateral SVZ and the number of neuroblasts and neurons probably generated from neural progenitor cells in the SVZ. These newly generated neuroblasts and neurons in the ischemic striatum after stroke have been proposed to migrate from the ipsilateral SVZ (Yamashita et al., 2006). Since the number of PSA-NCAM/BrdU cells was decreased in the ipsilateral SVZ and was increased in the ipsilateral striatum at 28 days compared with that at 14 days after ischemia, these time profile in our experiment indicates that newly generated neuroblasts migrated from the ipsilateral SVZ to injured striatum and some of them differentiated mature neurons in accordance with previous report (Yamashita et al., 2006).

The actions of Gal-1 in neurogenesis appear to be specifically mediated through carbohydrate binding. Gal-1 has previously been associated with many cellular functions, including development, proliferation, differentiation, immunity and apoptosis (Perillo et al., 1998; Liu, 2002; Camby et al., 2006). The extracellular functions require the carbohydrate-binding properties of Gal-1, while the intracellular functions are associated with carbohydrate-independent interactions between Gal-1 and other proteins (Perillo et al., 1998; Liu, 2002; Camby et al., 2006). Extracellularly, Gal-1 has the potential to bind to various surface receptors on a variety of cell types as well as extracellular matrix proteins, thus causing cell proliferation or apoptosis, modulating cell adhesion and inducing cell migration (Perillo et al., 1998; Liu, 2002; Camby et al., 2006). Intracellularly, Gal-1 can regulate cell transformation and cell motility (Liu, 2002; Camby et al., 2006). The proliferation of neural stem cells (Sakaguchi et al., 2006) and hematopoietic progenitor cells (Vas et al., 2005) is enhanced by carbohydrate binding by extracellular Gal-1. In our experiment, Gal-1 appears to regulate neurogenesis extracellularly since anti-Gal-1 Ab inhibited neurogenesis and Ox-Gal-1, which cannot bind carbohydrate, did not promote neurogenesis. These results suggest that the carbohydrate expressed on the NSPCs functions in the proliferation of NSPCs through its interactions with Gal-1. Extracellular Galectin-1 can bind to the carbohydrate structures on Integrin β1 (Gu et al., 1994; Hughes, 2001) and Laminin (Barondes et al., 1994; Gu et al., 1994; Zhou and Cummings, 1990), both of which are expressed by NSPCs and are important in their proliferation (Campos et al., 2004; Jacques et al., 1998). In non-neural tissues, the affinity of Integrin β1 for Laminin is increased or decreased by the direct binding of Galectin-1, probably depending on the subtypes of Laminins and/or Integrin α chains expressed in the cells (Hughes, 2001). Further studies should reveal whether Galectin-1 increases the affinity between Integrin β1 and Laminin, which may be an important step in NSPCs proliferation.

In the present study, an average of 6–12 BrdU/NeuN double positive cells in a striatal ROI field (0.3 × 0.3 mm, 10 μm section) were observed in the area of selective neuronal death around the striatal infarct even in non-treated ischemic animals. This density of newly generated neurons after focal ischemia is somewhat higher than what has been reported previously, although it is difficult to compare with previous experiments that have used different ischemia models, species and BrdU administration protocols (Arvidsson et al., 2002; Parent et al., 2002; Yamashita et al., 2006). The reason for this difference is unclear, but most previous experiments investigated the BrdU/NeuN double positive cells in the penumbral area (Ohab et al., 2006; Yamashita et al., 2006). In the present study, a gerbil model of repeated unilateral carotid artery occlusion was utilized, which in turn may cause a widespread lesion of selective neuronal death around the infarct in the ipsilateral cerebral hemisphere. The ischemic periphery signifies regions evolving selective neuronal death (Sun et al., 2006), which appears different from the penumbral region described in previous studies. After focal ischemia, blood flow is reduced to <15%, <40% and >40% of normal in the core, penumbral and extra-penumbral regions, respectively (Hossmann, 1994; Nedergaard et al., 1986). Given a long enough temporary insult, both the core and penumbra become infracted (Zhao et al., 1997), whereas the extra-penumbral zone only shows death of isolated neurons and less inflammation. Our results suggest that the widespread area of selective neuronal death may have a suitable environment for the survival of newly generated neuroblasts or neurons.

Gal-1 administration promotes functional recovery

In our experiment, intraventricular infusion of Gal-1 strongly influenced sensorimotor function after focal ischemia. CS-Gal-1 administration after focal ischemia markedly increased the

number of newly generated BrdU/PSA-NCAM-positive neuroblasts and BrdU/NeuN-positive neurons (Figs. 6B and C), mainly in the area of selective neuronal death in the ipsilateral striatum without a deficit in migration of BrdU-positive cells toward the OB (Fig. 6D). Although neurogenesis in the adult brain is related to neurological function (Gould et al., 1999), the question of whether neurogenesis improves functional recovery after stroke is still uncertain. However, several papers support the hypothesis that increased neurogenesis is related to functional neurological improvement after stroke, and treatment of stroke with growth factors or drugs promotes functional recovery concomitant with increased neurogenesis (Nakatomi et al., 2002; Zhang et al., 2002; Liu et al., 2007). Also, in our experiment, CS-Gal-1 administration led to functional recovery from sensorimotor disturbance, and anti-Gal-1 exacerbated ischemia-induced sensorimotor deficits. Spatial cognitive dysfunction evaluated by the T-maze test showed no difference among the experimental groups. In our previous studies using the same gerbil model, this cognitive function was markedly influenced by ischemic damage to the ipsilateral CA1 region of hippocampus or the ipsilateral visual cortex (Ishibashi et al., 2003). In our present study, however, no difference of the number of newly generated neuroblasts or neurons was detectable in the CA1 region of hippocampus and the visual cortex among the experimental groups (data not shown). Thus, we think that this enhanced neural progenitor production may play a role in neurological functional recovery. We have not demonstrated that these newly produced cells integrate into the injured local neural circuit and are electrically active. Therefore, the data associating neurogenesis with functional recovery should be interpreted with caution.

In previous experiments, Gal-1 administration provided a neuroprotective effect in a murine ALS model (Chang-Hong et al., 2005), and induced neurite-outgrowth in peripheral nerve injured rats (Inagaki et al., 2000; Horie et al., 2004). However, these experiments were carried out with oxidized Gal-1, which is known to lose its carbohydrate-binding activity. By contrast, the effects on neurological function in our focal ischemic model was observed only with CS (reduced)-Gal-1, but not with Ox-Gal-1. In terms of neuroprotective effect of Gal-1, we administered both Ox- or CS- Gal-1 at the acute phase of ischemic surgery, but no significant difference was detected in total infarct volume both 14 and 28 days after surgery among the treatment groups. These results indicate that Ox-Gal-1 or CS-Gal-1 could not provide a neuroprotective effect against ischemic injury in our focal ischemic model.

In galectin-1-null mice, neurite outgrowth and targeting of olfactory neurons was altered, demonstrating a role for Gal-1 in neural development (Puche et al., 1996). Promotion of neurite outgrowth was reported to improve neurological outcome after stroke (Wiessner et al., 2003). In our present study, therefore, neurite outgrowth-promoting activity induced by Gal-1 may contribute to a part of the functional recovery, although it remains to be elucidated whether CS-Gal-1 could promote the neurite outgrowth in the injured brain. Further investigations will be needed to understand fully these mechanisms of functional recovery in CS-Gal-1 animals after stroke.

In summary, we demonstrated that Gal-1 is an endogenous protein contributing to long-term plasticity after brain ischemia. We therefore propose Gal-1 as a potential new drug for brain infarction.

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