Accumulation of stress-related proteins within the glomeruli of the rat olfactory bulb following damage to olfactory receptor neurons*

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Summary. The expression of stress-responsive proteins, such as nestin and a 27-kDa heat-shock protein (HSP27), was immunohistochemically examined in order to demonstrate glial responses in the rat olfactory bulb following sensory deprivation. At 3 days to 1 week after sensory deprivation, numerous nestin-expressing cells appeared within the glomerulus of the olfactory bulb. These cells were regarded as reactive astrocytes since they were immunoreactive for glial fibrillary acidic protein and showed hypertrophic features. The glomeruli, in which nestin-immunoreactive astrocytes were localized, were filled with degenerating terminals of olfactory receptor neurons and migrated microglia. A small population of nestin-immunoreactive cells was positive for a proliferating cell marker, Ki67 (8.0–9.7% at 3 days; 3.1–5.0% at 1 week). At 3 weeks, nestin-immunoreactive astrocytes were occasionally detected.

At 6 weeks, when the olfactory receptor neurons had completely recovered, no nestin-immunoreactive astrocytes were detected. HSP 27 was also expressed within the glomerular astrocytes and showed a similar spatiotemporal expression pattern to nestin. The present study suggests that reactive astrocytes may be involved in axonal regeneration and synaptic remodeling in the olfactory system, through the recapitulation of developmentally regulated proteins, such as nestin and HSP27.

Introduction

The glomeruli of the olfactory bulb represent anatomical and functional entities in which the axons derived from the olfactory receptor neurons in the olfactory epithelium of the nose terminate to establish specialized synaptic interactions with dendrites of periglomerular, tufted, and mitral cells. This region of the brain is a unique region in adult mammals that exhibits plastic synapse formation, as the olfactory epithelium undergoes neurogenesis under physiological conditions and in response to lesions. Little is known about the role of glial cells in the synapse formation in glomeruli of the olfactory bulb, whereas an olfactory ensheathing cell, which is a unique type of Schwann cell enclosing axons of the olfactory receptor neurons within the olfactory nerve, is known to play a crucial role in promoting the long-distance growth of regenerating axons (Reviewed by Boyd *et al.*, 2003). Much attention has been paid to reports that discuss the active regulatory role of astrocytes, such as in inducing neurogenesis in adult neural stem cells (Song *et al.*, 2002) and in regulating synapse formation (Mauch *et al.*, 2001;

Received September 26, 2008

*This work was supported by a Health and Labour Sciences research grant (H16-kokoro-026) from the Ministry of Health, Labour and Welfare of Japan.

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Ullian *et al.*, 2001), rather than focusing on the supportive role that has traditionally been assigned to them. Thus, it is assumed that the astrocytes in this specific region of the olfactory bulb may also be involved in plastic synapse formation.

The aim of the present study is to explore the response of glial cells, especially astrocytes, in the sensory-deprived olfactory bulb. To this end, we immunohistochemically examined the expression of nestin and a 27-kDa heat-shock protein (HSP27), both of which are known to be developmentally regulated proteins—inducible under various situations of cellular stress and proliferation (nestin: Frisen *et al.*, 1995; Duggal *et al.*, 1997; Scorza *et al.*, 2005, HSP27: reviewed by Ciocca *et al.*, 1993; Plumier *et al.*, 1996; Costigan *et al.*, 1998; Lewis *et al.*, 1999), within the olfactory bulb of adult rats in which the olfactory epithelium had been injured by a single subcutaneous injection of diethyldithiocarbamate (DDTC) (Ravi *et al.*, 1997; Struble *et al.*, 1998). The results demonstrated that nestin-expressing reactive astrocytes subacutely appeared within the glomerulus (mainly at 3 days to 1 week post-injection) and that they mostly coexpressed HSP27. Double-immunolabeling with a proliferating cell marker, Ki67, showed that part of them demonstrated mitotic activity. The role of these reactive astrocytes, which coexpress nestin and HSP27 within the glomerulus, is discussed.

Materials and Methods

Subjects

Adult male Wistar rats weighing 200–350 g were used for all the experiments. The animals were anaesthetized with diethyl ether and received a single subcutaneous injection of 600 mg/kg of 1.04 M sodium diethyldithiocarbamate (DDTC) (Sigma Chemical, St. Louis, MO, USA) dissolved in 0.01 M phosphate-buffered saline (PBS) at pH 7.4. This protocol results in severe damage to the olfactory epithelium at a few days post-injection, followed by a subsequent regeneration and recovery to normal levels by 5 weeks post-injection (Ravi *et al.*, 1997; Struble *et al.*, 1998). Animals were sacrificed after 1, 3, and 4 days, and 1, 3, and 6 weeks. Control animals received a PBS injection, while two animals remained untreated, in order to ensure that no damage was caused to the olfactory system by the injection. The animals were transcardially perfused with PBS, followed by a mixture of 2.8% paraformaldehyde (PA) and 0.2% picric acid in a 0.1 M phosphate buffer (PB) at pH 7.4. The olfactory

bulb was removed intact and placed in 4% PA in PB overnight. The tissues were immersed in a 20% sucrose buffer overnight, embedded in Embedding Matrix, and immediately frozen at -80°C . The olfactory bulb was cut frontally into 40 μm -thick serial sections. The free-floating sections were immersed in a mixture of glycerin and 0.02 M potassium phosphate-buffered saline (KPBS) and kept at -20°C . The olfactory mucosa of the upper back portion of the nasal septum was also removed and processed in the same manner in order to check the reversible lesion of the olfactory epithelium and to examine nestin expression in olfactory ensheathing cells. The tissue was cut into 10 μm -thick serial perpendicular sections on a cryostat and mounted onto glass slides.

All of the experimental procedures were reviewed by the Committee on Ethics for Animal Experiments of the Faculty of Medicine, Kyushu University and carried out according to the Guidelines for Animal Experiments of the University, and Law No. 105 and Notification No. 6 of the Japanese Government.

Immunohistochemistry

The immunohistochemical procedure used in the present study has been described elsewhere (Hirata *et al.*, 2003; Yasuoka *et al.*, 2004). Briefly, non-specific binding sites were blocked by preincubation with 1% bovine serum albumin in KPBS with 0.1% Triton X-100 at 4°C for 1 h. For nestin immunohistochemistry, free floating serial sections of the olfactory bulb (240- μm interval) were first incubated with the primary antibody, a mouse mAb against nestin (Rat 401, developed by S. Hockfield and obtained from the Developmental Studies Hybridoma Bank [DSHB] maintained by The university of Iowa, Dept. of Biological Sciences, Iowa City, IA, USA 52242) (diluted 1:1,000 in KPBS) for 4 days at 4°C and then with the secondary antibody, a FITC-conjugated anti-mouse IgG (Jackson, PA, USA), overnight at 4°C . Control sections were processed identically and in parallel; however, these were incubated with KPBS instead of with the primary antibodies. No labeling was detected in these controls. The FITC-labeled sections were nuclear-stained with propidium iodide (PI) using a Vectashield mounting medium with PI (Vector, CA, USA). For cellular identification of the nestin-immunoreactive elements, a double-immunofluorescence procedure for nestin with GFAP was performed. Sections were incubated with a mixture of mouse mAb against nestin and a rabbit polyclonal antibody (pAb) against GFAP (DAKO, Denmark) (1:10), followed with a mixture of FITC-conjugated donkey anti-mouse IgG and Texas

Red-conjugated anti-rabbit IgG (Jackson) or a mixture of a biotinylated horse anti-mouse IgG (Vector) and Alexa488-conjugated anti-rabbit IgG (Molecular Probes, OR, USA), and finally with Texas Red-conjugated streptavidin (Jackson) for binding to the biotinylated secondary antibodies. The same procedure for nestin and/or GFAP was used on sections of the olfactory mucosa, except that the incubation conditions for each primary and secondary antibody were for 1 day at 4 °C and for 4 h at room temperature, respectively.

To demonstrate the relationship between nestin-immunoreactive glomerular astrocytes and other cellular elements—such as microglia and olfactory receptor neurons—and to compare the expression patterns of nestin and HSP27, a double immunofluorescence procedure was performed on sections of the olfactory bulb in the same way using a mixture of mouse mAb against nestin and either a rabbit pAb against Iba1 (a microglial marker) (Wako, Japan), a goat pAb against olfactory marker

protein (OMP) (a marker of olfactory receptor neurons) (Wako), or a goat pAb against HSP27 (Santa Cruz, CA, USA) as primary antibodies, followed by the appropriate secondary antibodies (Table 1).

To estimate the number of proliferating nestin-immunoreactive glomerular astrocytes, 5 serial frontal sections (240- μ m interval) through the middle part of the olfactory bulbs obtained from 3 rats at each time point were double immunofluorescence-labeled for nestin and Ki67 using a mixture of mAb against nestin and rabbit pAb against Ki67 (Yleiyi, Rome, Italy) as primary antibodies and then using a mixture of FITC-conjugated donkey anti-mouse IgG and Texas Red-conjugated anti-rabbit IgG as secondary antibodies. The sections were further nuclear-stained with DAPI using a Vectashield mounting medium with DAPI (Vector) (cf. Fig. 3E).

The sections were observed under a fluorescence light microscope (Zeiss Axioplan) and images were taken by a DP70 camera and stored on a computer.

Table 1. List of primary antibodies, secondary antibodies, and fluorophore-conjugated streptavidins which are utilized for binding to the biotinylated secondary antibodies.

	Company	Dilution	Host
Primary antibody			
Nestin	DBSH	1 : 1000	Mouse
HSP27	Santa Cruz	1 : 200	Goat
OMP	Wako	1 : 1000	Goat
GFAP	DAKO	1 : 10	Rabbit
Iba1	Wako	1 : 100	Rabbit
Ki67	Yleiyi	1 : 5	Rabbit
Secondary antibody			
FITC- anti-mouse	Jackson	1 : 200	Donkey
biotinylated anti-mouse	Vector	1 : 200	Horse
Alexa 488-anti-goat	Molecular Probes	1 : 1000	Donkey
Alexa 488-anti-rabbit	Molecular Probes	1 : 1000	Goat
Texas Red-anti-rabbit	Jackson	1 : 200	Donkey
Fluorophore-streptavidin			
Texas red-streptavidin	Jackson	1 : 400	
HRP-streptavidin	DAKO	1 : 100	

Confocal laser scanning microscopy (CLSM)

Double-fluorescence-labeled sections were imaged with a confocal laser scanning imaging system (LSM-GB200) attached to a microscope (Olympus). They were illuminated with an excitation wavelength of 488 nm (argon laser) for Alexa 488 and FITC, and 568 nm (krypton laser) for Texas Red and PI. To show the fine processes extending from the cell bodies, a series of optical sections at 1.5- μm intervals was projected and extended onto a single plane 10–20 μm in thickness (volume projection method) (cf. Fig. 2L–N and 4H–M). Green and red images were presented either as a superimposed image or separately as a grayscale image. The images were taken using $\times 4$, $\times 10$, $\times 20$, or $\times 40$ objective lenses. All figures are confocal images, unless described as fluorescent micrographs in the figure legend.

SEM

Scanning electron microscopy (SEM) was carried out in order to examine the repair processes of the olfactory epithelium. Animals were perfused with a mixture of 4% PA–0.05% glutaraldehyde (GA) in a 0.1 M cacodylate buffer (CB). The olfactory mucosa was removed and postfixed with 2% GA in PB. The tissue was dehydrated in a graded ethanol series, transferred to a graded t-butyl alcohol series, and freeze-dried. Tissue was mounted onto double-sided carbon tape and coated with osmium in an HPC-1S osmium coater before being observed through a scanning electron microscope (JSM-840, Japan) at an accelerating voltage of 8 kV.

Semithin sections of nestin-immunolabeled olfactory bulb

To clarify the morphological details of the nestin-immunoreactive glomerular astrocytes, animals were perfused with a mixture of 4% PA–0.05% GA in CB. The olfactory bulb was removed and cut frontally into 50 μm -thick sections with a Vibratome. The tissue was processed for nestin immunohistochemistry using diaminobenzidine visualization. Then the sections were postfixed in 2% GA in PB, followed by 1% OsO₄ in PB, before being embedded in Epon 812. Semithin sections were made and counterstained with 0.1% toluidine blue. The olfactory bulbs from control animals were processed in the same way, but without the nestin immunohistochemistry procedure.

Results

Time course of regeneration of olfactory receptor neurons

The combination of light microscopy of the PI nuclear staining (Fig. 1A–F) with SEM (Fig. 1G–J) was useful for demonstrating the regenerative process of the olfactory receptor neurons following the DDTC injection. In intact animals, the olfactory epithelium—which contains olfactory receptor neurons—is typically a tall pseudostratified epithelium of about 40 μm in thickness (Fig. 1A). At 1 day post-injection almost the entire olfactory epithelium had been removed, except for one or two layers of flattened cells covering the lamina propria (Fig. 1B). Subsequently, regeneration of the olfactory epithelium began and this gradually progressed (Fig. 1C–D). At 3 weeks post-injection, the height of the olfactory epithelium had almost reached the normal level; however, it showed an irregular nuclear arrangement (Fig. 1E). At 6 weeks post-injection, it showed a regular nuclear arrangement as in the intact olfactory mucosa (Fig. 1F). Observations of the luminal surface of the intact olfactory epithelium by SEM showed a unique apical process of mature olfactory receptor neurons, known as an olfactory vesicle (Nomura *et al.*, 2004). This possessed ten or more specialized cilia—the membrane of which is known to bear the receptors for odorants—extending horizontally in various directions (Fig. 1G). After the DDTC injection, the olfactory vesicles with specialized cilia completely disappeared. At 1 week post-injection, the surface was covered with fibrous structures although a few olfactory vesicle-like structures were occasionally detected (Fig. 1H). The olfactory vesicle-like structures markedly increased in number after 3 weeks; although they still showed an immature profile because they lacked cilia (Fig. 1I). At 6 weeks, mature olfactory vesicles with specialized cilia, similar to those of the control, reappeared (Fig. 1J). Thus, the olfactory receptor neurons within the olfactory epithelium had been completely repaired at 6 weeks post-injection.

Appearance of nestin-immunoreactive olfactory ensheathing cells within the olfactory mucosa after the DDTC injection

GFAP immunohistochemistry revealed a unique profile of olfactory ensheathing cells within the olfactory nerve (Fig. 2A). The cells showed no marked nestin expression within the intact olfactory mucosa (Fig. 2B). After the

Fig. 1. Fluorescent micrographs of PI-nuclear staining of the olfactory mucosa showing rapid damage to the olfactory epithelium after DDTC injection and subsequent gradual regeneration (A–F) and scanning electron micrographs showing the reformation of olfactory receptor sites (G–J). Note that reformation of the receptor structure is accomplished at 6 weeks (J) although the height of the epithelium appears to reach the control level earlier than at this time point. A and G: Controls. Arrows indicate olfactory vesicles. Scale bars = 10 μ m

Fig. 2. Simultaneous nestin expression in olfactory ensheathing cells of the olfactory mucosa and in the glomerular astrocytes of the olfactory bulb at 3 days and 1 week after DDTC injection. **A–G:** The olfactory mucosa, **H–N:** the olfactory bulb. **A:** GFAP (green) immunolabeling with PI nuclear staining (red) showing the unique morphology of the olfactory ensheathing cells in the olfactory nerve in the intact olfactory mucosa. **B:** Nestin (green) immunolabeling with PI nuclear staining (red) showing that the nestin expression is only seen in endothelial cells of a blood capillary in the intact olfactory mucosa. **C** and **D:** Nestin (green) immunolabeling with PI nuclear staining (red) showing that strong nestin-immunoreactive structures appear beneath the regenerating olfactory epithelium at 3 days (**C**) and 1 week (**D**) post-injection. **E–G:** Double-immunolabeling of nestin and GFAP of the olfactory mucosa at 1 week post-injection. The grayscale images of nestin (**E**) and GFAP (**F**) and their superimposed pseudocolor image (**G**) show that nestin expression is seen in some olfactory ensheathing cells. **A–D:** Fluorescent micrographs. Scale bar = 10 μm .

DDTC injection, strong nestin-immunoreactive structures appeared after 3 days to 1 week (Fig. 2C, D). Double-immunolabeling of nestin and GFAP confirmed that the nestin-immunoreactive structures corresponded to a part of the GFAP-immunoreactive ensheathing cells (Fig. 2E–G).

Appearance of nestin-immunoreactive astrocytes within the glomerulus after the DDTC injection

Double-immunolabeling of nestin and OMP showed that numerous nestin-immunoreactive cells appeared within the glomeruli of the olfactory bulb at 3 days (Fig. 2H, I) to 1 week post-injection (Fig. 2J), that is, at the early stage of regeneration in the olfactory receptor neurons, whereas no nestin-expression was seen within the glomeruli of the control olfactory bulb (Fig. 2K). The nestin-immunoreactive cells—which had a star-like profile—were located among scattered

OMP-immunoreactive dots implying the destruction of terminals of the olfactory receptor neurons after the DDTC injection (Fig. 2I). In contrast the glomeruli of the control olfactory bulb were only occupied by homogeneous OMP-immunoreactive ribbon-like structures (Fig. 2K). No nestin-immunoreactive cells were detected within the nerve fiber layer where the olfactory ensheathing cells exist (Fig. 2H, J). Double-immunolabeling of nestin and GFAP (Fig. 2L–N) revealed that the nestin-immunoreactive cells coexpressed GFAP, suggesting that they were astrocytes. The density of the nestin-immunoreactive astrocytes ranged from 37.0 to 61.7/section at 3 days (3 rats) and from 40.8 to 63.8/section at 1 week post-injection (3 rats). At 3 weeks after the DDTC injection, the nestin-immunoreactive astrocytes were detected within only a few glomeruli (cf. Fig. 4D). The density of the nestin-immunoreactive astrocytes ranged from 0.7 to 1.6/section at 3 weeks (3 rats). After that, they completely disappeared.

Fig. 2. **H–K:** Double-immunolabeling of nestin (red) and OMP (green) of the olfactory bulb at 3 days (**H** and **I**) and 1 week (**J**) post-injection, and the intact olfactory bulb (**K**). Note the OMP-immunoreactive scattered dots suggesting the degeneration of terminals of olfactory receptor neurons after DDTC injection (**I**). Asterisks in **H** indicate nestin-immunoreactivity in the subependymal zone. **I** is a higher magnification of the site indicated by the arrow in **H**. **L–N:** Double-immunolabeling of nestin and GFAP of the olfactory bulb at 3 days post-injection. The grayscale images of nestin (**L**) and GFAP (**M**), and their superimposed pseudocolor image (**N**) show that the nestin expression can be seen in some astrocytes. **J** and **K:** Fluorescent micrographs. Scale bars = 50 μm (**H**, **J**, **K**), 10 μm (**I**, **N**)

Double-immunolabeling of nestin and the microglial marker, Iba1, showed that the nestin-immunoreactive astrocytes were closely associated with Iba1-immunoreactive microglia that had an ameboid figure, which is characteristic of activated microglia (Fig. 3A, B). In toluidine blue-stained semithin-sections, the glomerulus of the intact olfactory bulb showed few cellular profiles except for the endothelial cells of the capillary (Fig. 3C). In contrast, the glomerulus of the sensory-deprived olfactory bulb, which had been immunostained for nestin, contained numerous migrated cells, including nestin-immunoreactive astrocytes and microglia, along with the debris of degenerating nerve elements (Fig. 3D). The nestin-immunoreactive astrocytes showed hypertrophic features with a large, round pale nucleus (about 10 μm in diameter) characteristic of reactive astrocytes. These were often located at the periglomerular region, and their tapering processes often ended at the blood capillary (Fig. 3D).

Semiquantification of proliferating nestin-immunoreactive astrocytes

The number of proliferating nestin-immunoreactive astrocytes was counted in serial frontal sections that had been triple-labeled with nestin, Ki67 and DAPI. Only the cells with a clear nucleus were counted (Fig. 3E). The proportion of nestin/Ki67-double-immunoreactive astrocytes to all nestin-immunoreactive astrocytes was 8.0–9.7% at 3 days, 3.1–5.0% at 1 week, and 0% at 3 weeks. This suggests that proliferation peaked at the first appearance of nestin-immunoreactive astrocytes before decreasing.

Coexpression of nestin and HSP27

Double-immunolabeling of nestin and HSP27 of the olfactory bulb clearly demonstrated the coexpression of the two proteins after the DDTC injection. Changes in

Fig. 3. Legend on the opposite page.

the expression pattern of the two proteins were readily observed by confocal images of the entire olfactory bulb (Fig. 4A–D). At 3 days post-injection, a considerable number of glomeruli expressed both nestin and HSP27 (Fig. 4B). At 1 week post-injection (Fig. 4C), the number of glomeruli expressing these two molecules appeared to be unchanged, except that the intensity of HSP27 in most glomeruli had decreased compared to that seen at 3 days. After 3 weeks, only a few glomeruli expressing the two molecules were found (Fig. 4D). Higher magnification showed that, after 3 days (Fig. 4E–G), almost all the nestin-immunoreactive astrocytes coexpressed HSP27, although the expression was too strong to delineate any cellular profile. After 1 week (Fig. 4H–J), almost all the nestin-immunoreactive astrocytes coexpressed HSP27. In nestin/HSP27-double-immunoreactive astrocytes, HSP27 immunoreactivity apparently filled the entire cytoplasm, so that the true hypertrophic configurations of nestin-containing cells and their fine processes were disclosed. Numerous fine processes appeared to be associated with each other within a glomerulus and some of them extended beyond the glomerulus toward the deeper layer (arrows in 4J). At 3 weeks after the DDTC injection (Fig. 4K–M), most of the fine processes had disappeared in the nestin/HSP27-double-immunoreactive astrocytes.

Discussion

In the current study, severe damage to the olfactory epithelium was seen at 1 day after a single subcutaneous injection of 600 mg/kg of DDTC in rats. Subsequently, the damaged olfactory epithelium progressively recovered, as was reported in a DDTC injection-model

(Ravi *et al.*, 1997, Struble *et al.*, 1998) and also in some other olfactory epithelium lesion models that used an axonal transection of the olfactory receptor neurons (Graziadei and Monti-Graziadei, 1978), inhalation of methyl bromide (Schwob *et al.*, 1995), or irrigation with ZnSO₄ (Williams *et al.*, 2004). The SEM observation revealed the recovery process of the receptor site of the olfactory receptor neurons. Numerous olfactory vesicles with immature profiles were seen at 3 weeks; these matured with the formation of specialized cilia, which bear receptors for odorants, at 6 weeks post-lesion. The above time points correspond with the reinnervation of the olfactory bulb at about 3 weeks after axonal transection (Graziadei and Monti-Graziadei, 1980; Doucette *et al.*, 1983) and restoration of synaptic contact with dendrites at 6 weeks (Doucette *et al.*, 1983). Thus, the complete recovery of the receptor region observed by SEM may imply the reformation of their synaptic contact with secondary neurons within the glomeruli.

The present study demonstrated the transient appearance of nestin-expressing cells in the olfactory bulb at the start of regeneration of the olfactory epithelium. Based on the findings from the double-immunolabeling of nestin/GFAP, nestin/OMP, and nestin/Iba1, and of semithin sections of the olfactory bulb immunostained for nestin, the nestin-immunoreactive cells were regarded as reactive astrocytes that had migrated to the degenerating terminals of olfactory receptor neurons where activated microglia had also aggregated. The mechanism of nestin induction is still not fully understood, but gliotrophic factors and other diffusible factors released from degenerating neurons or infiltrating inflammatory cells are thought to be possible triggering factors (Chen *et al.*, 2006). According to our findings, the nestin-expressing astrocytes were often located in the periphery of the

Fig. 3. Nestin-immunoreactive astrocytes intermingling with activated microglia in the glomeruli (A, B, and D) and proliferation of nestin-immunoreactive astrocytes (E). A and B: Double-immunolabeling of Iba1 (red) and nestin (green) of the olfactory bulb at 1 week post-injection. Note that nestin-immunoreactive astrocytes are closely associated with the Iba1-immunoreactive microglia that have migrated into the glomeruli (B). Scale bars = 10 μ m. C and D: Toluidine blue-staining of glomeruli of intact olfactory bulb (C) and the olfactory bulb at 1 week post-injection (D). Brown represents the nestin-immunoreactive site. Note the periglomerular localization of nestin-immunoreactive astrocytes (arrows) and the contact of their processes with blood capillaries (arrowheads). Asterisks indicate the nuclei of migrated cells, most of which are presumably microglia. Scale bars = 10 μ m. E: Double-immunolabeling of Ki67 (red) and nestin (green) with DAPI nuclear staining (blue) of the olfactory bulb at 3 days post-injection that was used for counting the number of proliferating nestin-immunoreactive astrocytes. An arrow indicates a Ki67/nestin-double-immunoreactive cell. A fluorescent micrograph. Scale bar = 10 μ m

Fig. 4. Legend on the opposite page.

glomeruli and extended fine long processes within the glomeruli or toward the deeper layers. Some of these astrocytes were proliferating. Valverde *et al.*, (1992), who studied the development of the olfactory bulb using mAb Rat-401, demonstrated that, during the early postnatal days, olfactory glomeruli became complete through the transformation of nestin-immunoreactive radial glial cells into periglomerular astrocytes. They also demonstrated that nestin expression within these cells had virtually disappeared by the end of the first postnatal month. Given that nestin-expressing reactive astrocytes after CNS injury are originally derived from a nestin-expressing population (Frisen *et al.*, 1995), it seems logical to consider that the nestin-immunoreactive reactive astrocytes within the glomeruli actually originate from the nestin-immunoreactive radial glial cells.

Intriguingly, nestin expression was usually not detected within the nerve fiber layer of the olfactory bulb—where an olfactory ensheathing cell encloses the axon of the olfactory receptor neurons—although a simultaneous nestin expression was detected in some of the olfactory ensheathing cells within the nasal mucosa close to the damaged olfactory epithelium. This finding may reflect a unique property of the olfactory ensheathing cells, which maintain continuously open channels to allow for regrowth of olfactory nerve fibers without proliferation or migration, even though their enclosing axons are damaged (Li *et al.*, 2005).

Of particular interest is the fact that HSP27 was also induced in reactive astrocytes almost concurrently with nestin. HSP27, a member of the small heat-shock protein family, is developmentally regulated (Costigan *et al.*,

1998) and can be neuroprotective in the event of heat shock or other injuries (reviewed by Ciocca *et al.* 1993; Lewis *et al.*, 1999; Plumier *et al.*, 1996). HSP27 also participates in cytoskeletal dynamics by stabilization of the actin filaments that protect cells from insult (Lavoie *et al.*, 1995) or via the assembly of the intermediate filament protein which functions as a molecular chaperone (Perng *et al.*, 1999). This raises the possibility that HSP27 is also involved in the synthesis of the embryonic intermediate filament component, nestin. The coexpression of nestin and HSP27 within reactive astrocytes in cerebral abscesses was reported by Ha *et al.* (2002), who surmised that the increased synthesis of nestin was probably associated with small HSP synthesis via the MAP kinase-associated pathway.

Accumulating evidence gained mainly by *in vitro* studies has revealed a previously unknown function of astrocytes: they may actively participate in synaptic plasticity (Mauch *et al.*, 2001; Ullian *et al.*, 2001). In addition, in injury models, nestin induction in reactive astrocytes has been proposed to be involved in local remodeling and repair of the mature brain, including the facilitation of synapse formation related to neural plasticity (Frisen *et al.*, 1995; Duggal *et al.*, 1997; Krum and Rosenstein 1999; Scorza *et al.*, 2005). In the present study, reactive astrocytes with a coexpression of nestin and HSP27 appeared within the synaptic region of the olfactory bulb following sensory deprivation. Although their precise role still needs to be clarified, they may be involved in repair and synapse reformation. Recapitulation of these proteins suggests a structural plasticity that is thoroughly prepared for the continuous

Fig. 4. A–M: Coexpression of nestin and HSP27 in glomeruli after DDTC injection. Double-immunolabeling of nestin (red) and HSP27 (green) of the intact olfactory bulb (A) and the olfactory bulb at 3 days (B), 1 week (C) and 3 weeks (D) post-injection. Note that the coexpression of the two proteins (yellow) is limited to the glomerular layer (B, C). Higher magnifications of the white boxes in B, C, and D are shown in E–G, H–J, and K–M, respectively. The gray scale images of nestin (E, H and K) and HSP27 (F, I and L) and their superimposed pseudocolor images (G, J and M) are shown. Arrows (H–J) indicate a long process extending across the glomerular region toward the deeper layer. Scale bars = 100 μm (A–D), 20 μm (G), 10 μm (J, M)

processing of olfactory information. These findings provide fundamental data for a further understanding of the mechanisms underlying axonal regeneration and synaptic remodeling in the olfactory system.

Acknowledgements

We thank Mr. Fumitake Kadoya for his expert technical assistance. The English language in this manuscript was revised by Miss K. Miller (Royal English Language Centre, Fukuoka, Japan).

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