Localization of Nestin in Amygdaloid Kindled Rat: An Immunoelectron Microscopic Study

Toshitaka Nakagawa, Osamu Miyamoto, Najma A. Janjua, Roland N. Auer, Seigo Nagao, Toshifumi Itano

ABSTRACT: ***Background:*** Nestin is a class VI intermediate filament protein, expressed during early embryonic development in mammals. Postnatally, nestin and its mRNA are down-regulated and gradually disappear. Recently, nestin expression has been detected in the adult nervous system, and it has been suggested that this protein may be related to neurogenesis, although, its role in the mechanism of neurogenesis is not known. ***Methods:*** The present study examined the localization of nestin in CNS tissue of the amygdaloid kindled rat by light and electron microscopy. ***Results:*** Kindled animals showed nestin expression mainly in the piriform cortex and the perirhinal cortex. By light microscopy, nestin was shown to be expressed in astrocytes, neurons, and endothelial cells. Electron microscopy demonstrated nestin expression in endothelial cells, astrocytic perivascular end feet, the rare pericyte, neurons and oligodendrocytes. ***Conclusion:*** We conclude that epilepsy causes widespread nestin expression in many cell types in the CNS, including non-neural cells.

RÉSUMÉ: Localisation de la nestine chez les rats amygdaloid-kindled: une étude immunologique par microscopie électronique. ***Introduction :*** La nestine est une protéine associée aux filaments intermédiaires de classe VI exprimée tôt pendant le développement embryonnaire chez les mammifères. Après la naissance, la nestine et son ARNm sont régulés à la baisse et disparaissent graduellement. L'expression de la nestine à été détectée récemment dans le système nerveux adulte et on a suggéré que cette protéine pourrait être en relation avec la neurogenèse, bien que son rôle dans le mécanisme de la neurogenèse ne soit pas connu. ***Méthodes :*** Nous avons déterminé la localisation de la nestine dans le système nerveux central (SNC) du rat amygdaloid-kindled par microscopie optique et microscopie électronique. ***Résultats :*** L'expression de la nestine chez ces animaux était localisée principalement au niveau du cortex piriforme et du cortex périrhinal. À la microscopie optique, la nestine était exprimée dans les astrocytes, les neurones et les cellules endothéliales. À la microscopie électronique, la nestine était exprimée dans les cellules endothéliales, les terminaisons périvasculaires des astrocytes, de rares péricytes, les neurones et les oligodendrocytes. ***Conclusion :*** L'épilepsie cause une expression de la nestine dans plusieurs types de cellules du SNC, dont des cellules autres que des neurones.

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Nestin was discovered as an epitope in cells of the neural tube, identified by the monoclonal antibody RAT-401.[1] The cDNAof rat nestin has been cloned and the predicted amino acid sequence of the nestin gene indicates that it belongs to the intermediate filament (IF) family of proteins.[2] However, the sequence of nestin does not resemble any of the five known classes of IF proteins and, therefore, it has been designated a class VI IF protein,[2] with a molecular weight of about 220 kilodaltons.[3] During the early embryonic developmental stages, nestin is transiently expressed in multipotential neural precursor cells,[4,5] epithelial cells,[6] radial glia,[7] germinal matrix cells,[7] and vascular cells in the central nervous system (CNS).[8] However, postnatally, the nestin protein itself and its mRNA are down-regulated and gradually disappear.[4,7,9,10] In normal adult CNS, nestin is detected in limited regions such as the subventricular zones and olfactory bulb.[11-13] Neurogenesis has been shown to occur from the ependymal cells near the ventricles.[11] The

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immature neural precursors divide in a discrete zone of proliferation and then migrate while differentiating into neurons appropriate to their final location.[10,12,14]

In the adult hippocampus, the progeny of proliferative cells accumulates within the subgranular zone of the dentate gyrus.[15] Most of these cells are negative for mature neuronal or glial markers but some may express markers, including nestin, common to immature neurons.

The amygdaloid kindled rat is a well-established model of temporal lobe epilepsy.[16-19] To examine the remodeling process in kindling epilepsy, we studied nestin expression immunohistochemically in the amygdaloid kindled rat brain.[20] In the piriform cortex (PC) and perirhinal cortex (PRh) in fully kindled rats, double immunostaining including glial fibrillary acidic protein reveals that most of the reactive astrocytes in the PC express nestin.[20] We also show here that after epilepsy, there are nestin immunoreactive vascular cells, in addition to the astrocytes.

MATERIALS AND METHODS

Production of rat kindling model

Male Sprague-Dawley rats weighing 250-300 g were used. Animals were housed under a natural light-dark cycle with food and water *ad libitum*. All animal experiments were done in accordance with the Guiding Principles for the Care and Use of Animals approved by the Council of the Physiological Society of Japan.

Rats were anesthetized with nembutal sodium (10 mg/kg i.p.) (Abbott Laboratories, North Chicago, IL). Bipolar electrodes were implanted into the left amygdala (A -0.2, L 4.5, V 8.2mm, from bregma).[17,19,21] Three to four days after surgery, rats were stimulated twice a day at 9:00 and 15:00 hours for seven days a week with a biphasic square wave pulse (100-200 μampere peak to peak, 60 Hz, for one second) using two electric stimulators (NEC San-ei, Co. Ltd., Tokyo, Japan) to generate the biphasic pulse. The amplitude of the pulse was adjusted individually to subthreshold stimulation with signs of no immobility or mouth movements. In the kindled animals, the response to succeeding stimulation trials was evaluated at five kindling stages as described by Racine et al[22] with slight modifications[17,20,23] as follows: stage C1) immobility and/or rhythmic mouth movements; stage C2) C1 features plus contralateral head turning; stage C3) C2 features plus contralateral forelimb clonus; stage C4) C3 features plus generalized tonic-clonic seizures; and stage C5) C4 features plus generalized clonic seizures and falling over. We chose stage C5 as the stage after kindling. Stimulations were stopped after five successive seizures of stage C5 (group C5, n=10). A sham group (Group C0, n=10) was implanted with electrodes and handled identically, but did not receive an actual electrical stimulus.

Light and electron microscopy

Two hours after the last stimulus, the rats of the group C5 were deeply anesthetized with nembutal sodium solution (30 mg/kg i.p.) and perfused transcardially with 0.1 M phosphate buffer saline (pH 7.4) followed by a fixative consisting of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 20 min. The perfusion pressure was 150 cm H_2O. The brains were removed and post-fixed for three days in the same solution, and then cut into 20 μm frozen sections.

For histological examination, only free floating frozen sections were used. The sections were dried completely and put in 0.3% Triton X-100 (Sigma, St. Louis, MO) in 0.01 M phosphate buffered saline (PBS) for 1h. To quench endogenous peroxidase activity, the sections were put in 2% H_2O_2 in 0.01 M PBS for 30 minutes. After washing with 0.01M PBS, nonspecific reactions were blocked with 5% skim milk solution. A monoclonal antibody against nestin (RAT-401, developed by Susan Hockfield and obtained from the Developmental Studies Hybridoma Bank under the auspices of the National Institute of Child Health and Human Development and maintained by University of Iowa, Department of Biological Sciences, Iowa City) in 1% skim milk solution in 0.01 M PBS (1:100) was then applied to sections overnight at 4ºC. This antibody is known to recognize nestin in rat and mouse,[24,25] and its specificity has already been determined.[26] Sections were then incubated with a biotinylated second antibody for 30 minutes, washed well with PBS and then reacted with a mixture of 3,3'-diaminobenzidine (DAB) 1 μg/ml and H_2O_2 0.5% solution in PBS. After the DAB-peroxidase reaction, sections were post-fixed with 2% osmium tetroxide dissolved in PBS, for two hours at room temperature, dehydrated gradually in ethanol and embedded in Epon 812 resin (TAAB, Berkshire, UK) with slight modification.[20] For electron microscopy, ultra-thin sections were cut to 70-90 nm with an ultramicrotome (OmU4 Reichert, Vienna, Austria), and examined with a JEM-1200EX electron microscope (JEOL, Tokyo, Japan).

RESULTS

Light microscopic images from group C5 are shown in Figure 1. Nestin positive immunoreactivity was observed in PC and PRh (Figure 1A). Other regions showed no specific immunoprecipitate, except at the edges of the subventricular zone and the third ventricle (data not shown). The sections from rats that were implanted with electrodes but received no stimulations (Group C0) showed no staining (data not shown). The control sections from the group C5 that were prepared with normal IgG instead of RAT-401 also showed no immunostaining (data not shown). Staining in the PC and PRh of the group C5 showed the presence of astrocyte-like cells (Figure 1B). These cells were double stained by glial fibrillary acidic protein as shown in our previous study.[20] Neurons (Figure 1C) and some endothelial cells (Figure 1D) were also stained by RAT-401.

Electron microscopic images from groups C0 and C5 are shown in Figure 2. The sections from group C0 showed no immunoreactivity for nestin (Figure 2A). However, the endothelial cells and astrocytic end-feet of group C5 were stained by RAT-401 (Figure 2B-D). To verify the specific nature of the staining, three adjacent sections are shown in Figures 2B-D. The endothelial cells of smaller vessels of diameters around 8 μm were well-stained, in comparison to larger vessels. However, pericytes were rarely stained (Figure 2B). The endothelial cells with nuclei that wrapped around the lumen of the vessel were unstained (Figure 2). Around the microvessels only the endothelial cells or the astrocytic end-feet were stained in some sections (Figures 3A and B). Figure 4 shows higher magnification of nestin localization in and around the

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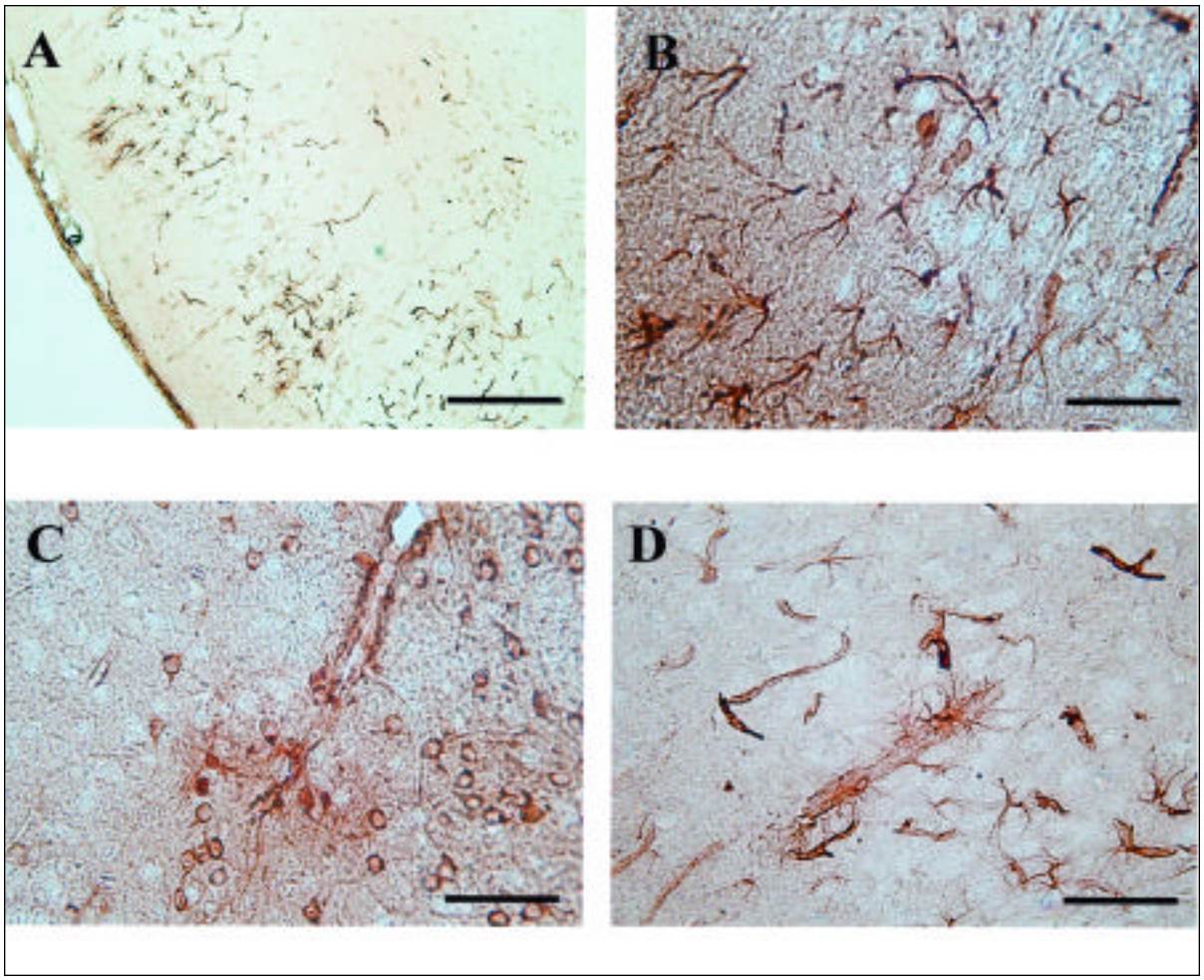

Figure 1: *Light microscopic images showing nestin expression in group C5. Scale bars, A: 400 μm, B to D: 100 μm. A and B show the immunopositive astrocytes. C shows neurons and oligodendrocytes, and D shows endothelial positivity.*

microvessels. Nestin localization in astrocytes are shown in Figure 5. The cytoplasm of neurons was also stained by nestin antibody (Figures 6A and B). Punctate staining of perikaryal cytoplasm was interpreted as filament cross-sections (Figure 6B). Some oligodendrocyte cytoplasm was also stained (Figures 6C and D).

Discussion

We here provide direct evidence, by light and electron microscopy, of nestin expression in neurons and glia of the PC after electrically kindled epilepsy in the rat. This study suggested the possibility that some initiating stimulus from the periphery or the CNS may trigger concurrent recruitment of endothelium. In fact, several other studies have shown nestin positivity in vascular cells.[3,8,27,28] The physiological interpretation of the localization of nestin in the vascular cells is still unclear. Although some light or confocal microscopic studies have shown localization of nestin around vessels,[3,8,15] there are several critical points in the assessment and interpretation of the data. One is the possibility of artifacts. If the transcardiac perfusion is not complete, peroxidase can be retained and some nonspecific staining may be observed. To avoid this artifact, we employed a specific staining in both experimental and sham or control groups. Careful observations were done of the sections from group C0 and of the normal IgG staining in group C5. Importantly, staining was observed in neither sham nor IgG treated sections.

Another critical problem in the evaluation of nestin expression data concerns the specific cellular localization of staining. Endothelial cells, perivascular cells, and astrocytic end-feet are difficult to distinguish by light microscopic analysis. Although studies have shown co-localization of nestin positive cells in the vessels in adult brain by electron microscopy,[27,28] the data have been inconsistent. Frisen et al[27] showed nestin staining in endothelial cells by the post- embedding immunogold method, but no clear data were presented about the astrocytic end-feet. On the other hand, Krum and Rosenstein[28] demonstrated nestin in the astroglial end-feet by the pre-embedding peroxidase method, but they did not detect any immunostaining in the endothelial cells. Pre- and post methods each have advantages.

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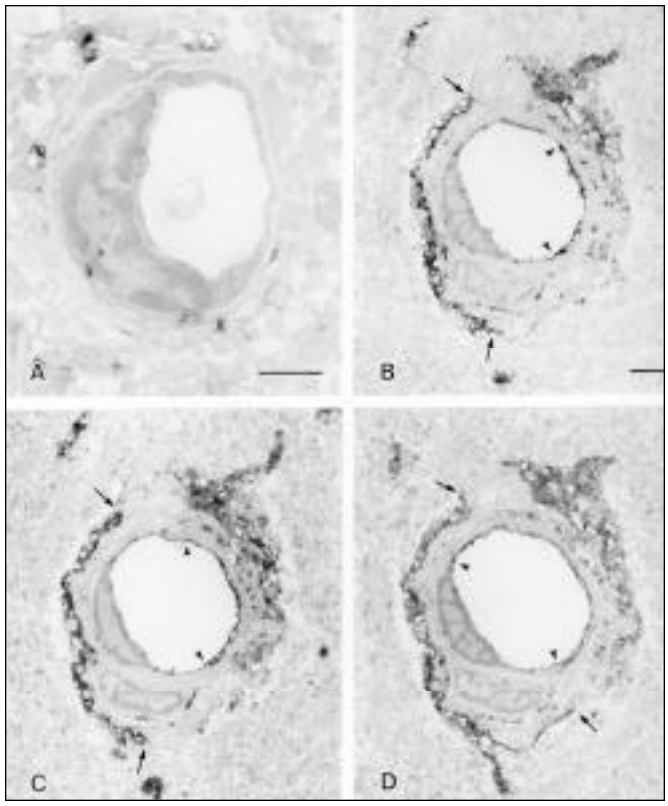

Figure 2: *Electron microscopic images showing nestin expression in and around microvessels in groups C5: A: group C0; B to D: adjacent sections from group C5. Arrowheads indicate an endothelial cell, and arrow indicates the astrocytic end foot, both of which show immunopositivity. Scale bars, A:1 μm, B to D: 2 μm.*

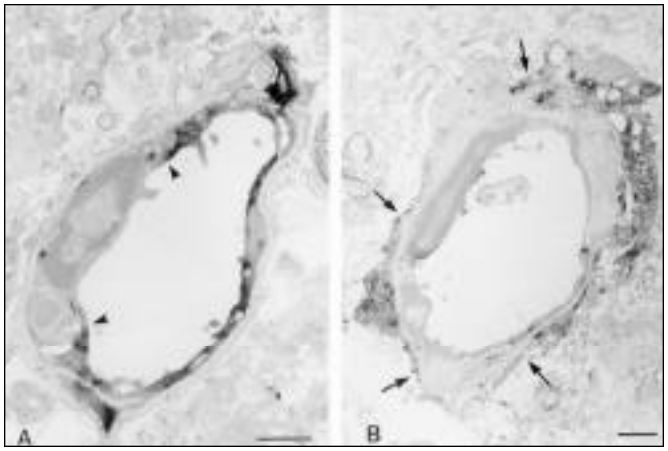

Figure 3: *Nestin expression in (A) or around (B) a microvessel in group C5: Arrowheads (A) indicate endothelial cell cytoplasm, and immunopositivity is seen in astrocytic end feet. Scale bar, 1 μm.*

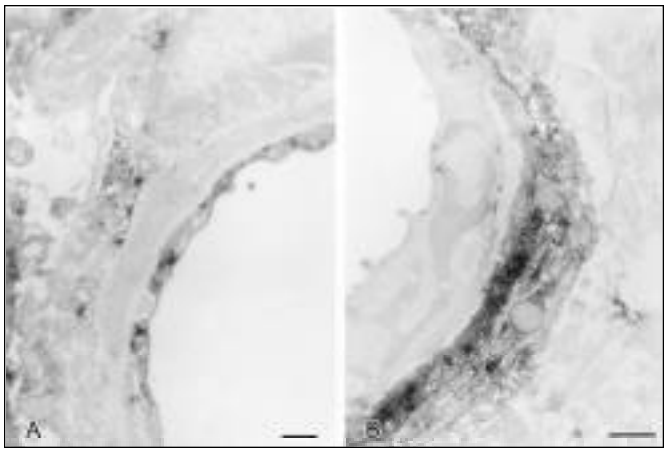

Figure 4: *Higher magnification of nestin expression in (A) or around (B) a microvessel in group C5: Scale bars, 2 μm and 0.5 μm for (A) and (B) respectively.*

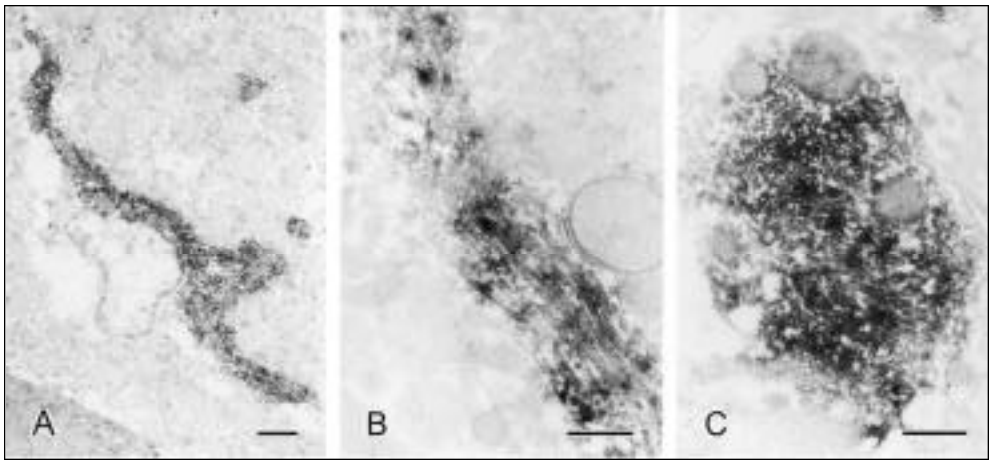

Figure 5: *Nestin expression in astrocytes (A) in group C5. (B) shows longitudinal section and (C) shows cross section of intermediate filaments. Scale bar, 2 μm in (A), 0.5 μm in (B) and (C).*

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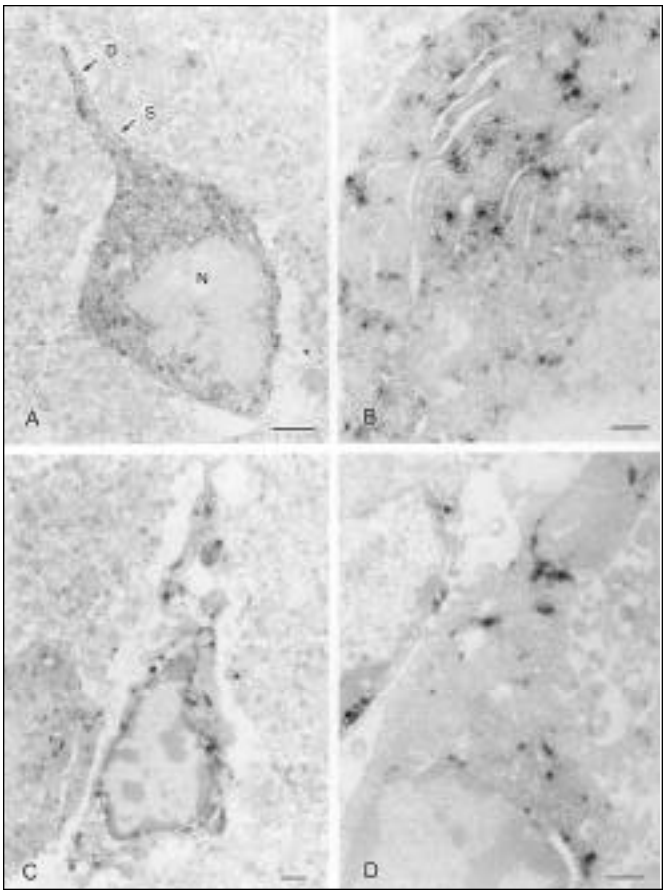

Figure 6: *(A) Electron microscopic images showing nestin expression in a neuron in group C5. N: nucleus, S: synapse, D: dendrite. (B) Punctate immunopositivity is seen in neuronal cytoplasm. Scale bar, 2 μm in A, 0.5 μm in B. (C-D) Electron microscopic images showing oligodendrocyte nestin expression in group C5: Scale bar, 1 μm in C, 0.5 μm in D.*

For example, pre-embedding retains more antigenicity, while post-embedding is superior for fine structural analysis. In the present experiments we chose the pre-embedding method, as one of the purposes of this study was to clarify the nature of the cytologic staining.

The role of nestin expression in the endothelial cell is still unknown. However, Palmer et al[15] recently reported that adult neurogenesis occurs within an angiogenic niche. They proposed two models of neuroangiogenic recruitment mechanism: signals originating from somatic tissues (Model I); or from CNS (Model II). In either model, certain cues may act to simultaneously stimulate neurogenesis and angiogenesis. Although the authors interpreted the presence of neural and endothelial precursors as a simple anatomic co-localization,[15] their data did not eliminate the possibility that these cells might be derived from a common precursor.

We detected nestin immunoreactive cells in the ventricular and subventricular zones, as described in another study of the normal adult rat.[4] We also detected nestin immunoreactive cells in PC and PRh but not in hippocampus, in stage C5 of the epilepsy. In the hippocampus, the nestin immunoreactive cells were too faint to be considered specific staining. Furthermore, some studies suggested that the activation of hippocampus is not necessary for the induction of focal seizures.[29,30]

Another critical point in the evaluation of nestin expression in PC is the specific response to the particular stimulus used. Recently, Loscher and Ebert[31] proposed that specific regions within PC and PRh function as critical links in the propagation of limbic seizures evoked from the area tempesta. Our previous study[20] showed that nestin expression was limited in the ipsilateral PC at the C3 stage of epilepsy. However, at stage C5, nestin immunoreactivity was observed bilaterally in the PC, especially in layer III, demonstrated electrophysiologically to be highly susceptible in terms of postkindling after discharge threshold.[31] According to the data, nestin expression may be related to electrical stimulation during the kindling epileptogenesis.

Nestin expression in adult brain after insults such as trauma, ischemia, epilepsy and ganglioglioma in several reports has been shown in astrocytes.[14,26,28,32,33] The present study observed nestin expression in three types of cell. Chronologically the expression in astrocytes is the first to occur, at the C3 stage ipsilaterally, neuronal expression being second to be expressed, at the C5 stage, and expression at the oligodendrocyte stage following last. Not only the time course, but also the staining pattern in the three types of cells is different. The strong and clear staining of the endothelial cell and astrocytic end-feet contrasts with the weaker, less distinct staining of the neural and oligodendrocytic elements. Also, punctuate staining patterns were observed only in neurons and oligodendrocytes. These data suggest that the localization and the roles of the intermediate filament in each cell might be different. Recently, Palmer et al[15] reported that the mammalian CNS contains multipotent stem cells that develop into neurons, astrocytes and oligodendrocytes in and around the ependyma or ventricle, suggesting nestin might play a role, albeit different, in regenerative efforts in each of these cell types.

Several studies have revealed nestin expression in the adult brain after an insult such as focal cerebral ischemia,[32] traumatic brain injury,[26,27,34] and neural transplantation.[27,28] After an insult, nestin immunoreactivity in glial, neural, and ependymal cells is reminiscent of protein expression in developing brain,[32] in that it is evanescent, disappearing several weeks after the insult.[28] These observations in the adult brain suggest that nestin expression might be closely related to neurogenesis after an insult,[32] but such a relationship would be difficult to prove directly. Together with results of the present study, the data also support the hypothesis that the PC may be the main region of recruitment and amplification in epileptogenesis.

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