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Postnatal development of nestin positive neurons in rat basal forebrain: Different onset and topography with choline acetyltransferase and parvalbumin expression

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A B S T R A C T

Our previous studies identified a sub-population of cholinergic neurons which express nestin in the rostral part of the basal forebrain (BF) in normal adult rats. In the present study, the postnatal developmental patterns of nestin, choline acetyl transferase (ChAT) and parvalbumin (PV) positive neurons were explored by means of immunohistochemistry combined with immunofluorescence double label methods. Compared with early onset of ChAT expression (from P1) and delayed onset of PV expression (from P16), nestin positive activity was detected in the BF from P9 and co-expressed by parts of the ChAT positive neurons within the same region during the whole postnatal development process. However, ChAT and PV were not coexpressed by the neurons within the medial septum-diagonal band of Broca (MS-DBB) of BF. These results might imply a composite of separate development patterns displayed by different subpopulations of cholinergic neurons (nestin positive cholinergic neurons and nestin negative cholinergic neurons) within this region. Moreover, the topographic distribution of nestin, ChAT and PV positive neurons also showed different characteristics. In summary, our present study revealed a remarkable timing and topographic difference on the postnatal development of the nestin expression within the MS-DBB of BF compared with ChAT and PV expression. It is further suggested that nestin is re-expressed by cholinergic neurons in the BF after differentiation but not persisted from neuronal precursor cells.

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1. Introduction

Basal forebrain composes a diverse group of telencephalic structures situated on the medial and ventral aspects of the cerebral hemispheres, which have received extensive attention for their involvement in the pathophysiology of Alzheimer's disease (Mesulam et al., 1983; Lawrence and Sahakian, 1995; Semba, 2000; Schliebs, 2005; Schliebs and Arendt, 2006). Cholinergic neurons, which express choline acetyltransferase (ChAT), are a major neuron subset generated in the basal forebrain and GABAergic neurons rank the second. They formed main component of septohippocampal projection (Gritti et al., 2003; Sotty et al., 2003; Wang et al., 2006).

Nestin is a cytoskeletal intermediate filament protein. It appears prenatally, marks immature cells and is re-expressed in mature reactive astrocytes (Hockfield and McKay, 1985; Lin et al., 1995; Duggal et al., 1997). However, in our previous studies, we identified a cluster of nestin-immunoreactive (nestin-ir) neurons in the rostral part of the basal forebrain (medial septum-diagonal band of Broca, MS-DBB) in normal adult rats and human (Gu et al., 2002; Wang et al., 2006). These nestin-ir neurons co-express neuron-specific enolase (NSE) and NeuN (a neural marker), but not glial fibrillary acidic protein. It is suggested that these nestin-ir cells are mature neurons. Untill 2011, the Hendrickson's study (Hendrickson et al., 2011) also proved our result that nestin-ir neurons existed on the BF. Next our result of double immunofluorescence labeling combined with single-cell RT-PCR certified that they are a subgroup of choline acetyl transferase positive neurons (Guo et al., 2010). Retrograde tracing revealed that a significant portion of these nestin positive cholinergic neurons projected to the hippocampus (Wang et al., 2003; Li et al., 2006) and the cell number and morphological characters present age related and aged memory deficit changes (Li et al., 2008). The developing patterns of nestin-containing neural precursors of the embryonic and postnatal CNS have been well documented (Dahl, 1981; Hockfield and McKay, 1985; Liem, 1993; Dahlstrand et al., 1995; Doetsch et al., 1997; Gubert et al., 2009).

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However, nestin expression and patterns of morphogenesis in postnatal BF remains to be delineated. It is not clear whether nestin expression within the BF neurons was the persistent expression from neuronal precursor cells to mature neurons or re-expression by neurons after differentiation. It is also necessary to determine if there are different expressing patterns between nestin and other functional proteins within this region.

The purpose of the present work is to describe the postnatal development of nestin positive neurons in BF and compare the temporospatial differences with ChAT positive cholinergic neurons and GABAergic neurons by means of immunohistochemistry employing antibodies against nestin, ChAT and parvalbumin (PV), which is know to be contained in GABAergic septohippocampal projection neurons. Immunofluorescence double label methods were used to detect if nestin was specific expressed in cholinergic neurons in both early postnatal and adult BF.

2. Materials and methods

2.1. Experimental animals

Exactly timed pregnant female Sprague-Dawley rats were obtained from Experimental Animal Center of Zhongshan School of Medicine, Sun Yat-sen University. The pups of our own breeding colony were utilized for this study, ranging from P1 to P28 (5 animals at each postnatal day) and P60 (5 animals). The day of birth was designated as the first postnatal day. All experimental procedures were performed in accordance with the guidelines of the ethical treatment of experimental animals of the Science and Technology Bureau of Guangdong Province which consistent with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23).

2.2. Immunohistochemistry procedures

All animals tested in the study were terminally anesthetized by an intraperitoneal injection of pentobarbital (40 mg/kg) then perfused intracardially with heparin saline (0.1% heparin in 0.9% saline) followed by paraformaldehyde [4% in phosphate-buffered saline (PBS); 100 ml/rat for 1–28 day rats and 250 ml/rat for 60 day rats]. After perfusions, the forebrains were removed and postfixed for 4 h at 4 °C in the same 4% paraformaldehyde solution. The blocks were then equilibrated in sucrose (30% in PBS), sectioned (35 μm) on a freezing microtome, and for every five sections, one was collected. A total of six series sections were collected per subject. From the first to fifth serial sections were processed for single immunohistochemical staining of nestin, ChAT, PV and double immunofluorescence staining of nestin with ChAT, ChAT with PV, respectively, the sixth serial sections were backed up.

Sections were treated for 10 min in 0.3% hydrogen peroxide, washed three times in PBS, and incubated in 1% bovine serum albumin (containing 0.3% Triton X-100 in PBS) for 30 min prior to overnight incubation at 4 °C with the primary antibody (nestin: mouse monoclone, Pharmingen, 1:800; ChAT: rabbit multiclone, Chemicon, 1:1000; PV: mouse monoclone, Sigma, 1:5000). After a 3 × 10 min rinse in PBS, the sections were incubated in biotinylated secondary antibody (goat anti-mouse or goat anti-rabbit IgG; Sigma, 1:200) at room temperature for 2 h. The sections were rinsed 3 × 10 min in PBS and incubated in avidin-biotin peroxidase complex (ABC kit, Sigma; 1:200) for 2 h at room temperature. Following thorough rinsing with PBS, staining was visualized by incubation in 3,3′-diaminobenzidine solution. After immunohistochemistry staining, floating tissue sections were mounted on superfrost plus glass slides, dehydrated, cleared, and coverslipped.

For the control experiments, the primary antibody was replaced with normal mouse serum (for nestin/PV immunocytochemistry) or normal rabbit serum (for ChAT immunocytochemistry). Nestin, ChAT and PV immunoreactive neurons were not found in the control sections.

2.3. Immunofluorescence double staining procedures

Sections were washed in PBS, and incubated in 1% bovine serum albumin (containing 0.3% Triton X-100 in PBS) for 30 min prior to overnight simultaneous incubation at 4 °C with two primary antibodies: nestin + ChAT/PV + ChAT; after a 3 × 10 min rinse in PBS, the sections were simultaneously incubated in corresponding Cy2 conjugated-goat anti-mouse IgG (Jackson ImmunoResearch, 1:100) and Rhodamine conjugated-goat anti-rabbit IgG (Jackson ImmunoResearch, 1:800)at room temperature for 2 h. After being washed, the sections were mounted on gelatin-coated glass slides, and coverslipped in 0.01 M PBS containing 50% glycerin, After staining, all the fluorescence-labeled sections were observed with Leica fluorescence microscope under appropriate filters for Cy2 (excitation 492 nm; emission 510 nm), Rhodamine (excitation 550 nm; emission 580 nm), respectively.

2.4. Estimate of the number of nestin, ChAT and PV positive neurons

The number of nestin, ChAT and PV-ir neurons in the MS-DBB were counted in rats at P1, P7, P9, P16, P21, P28 and P60. Counts of the nestin, ChAT and PV-ir neurons were obtained from four representative coronal sections that contained the rostral portion [medial septum (MS) and vertical limb diagonal band (vDB)] and caudal portion [horizontal limb diagonal band (hDB)] of the basal forebrain in every set slice. One horizontal line through the superior border of bilateral anterior commissure was utilized to define the boundary of MS and DBB. The neurons were calculated to MS if they touched the horizontal line through the superior border of bilateral anterior commissure. Below the horizontal line were calculated to DBB (vDB and hDB). The number of stained nestin, ChAT and PV positive neurons in the whole MS-DBB region were counted. The quantification of the neuronal soma body area at P1, P9, P16, P28 and P60 were performed using the image J 1.47 software.

All values were presented by means ± SD. Statistical analysis was performed using one-way ANOVA followed by the least significant difference *t*-test (LSD-*t*) for differences among groups.

3. Results

3.1. Immunohistochemistry staining

Fig. 1 shows the topographical distribution of nestin, ChAT and PV positive neurons within the BF in different postnatal day.

No nestin positive neuron was observed before day 8 of postnatal life (P8) (Figs. 1 and 2) in the BF. At P9, a weak neuronal nestin positive activity was detected in the MS-DBB. These neurons were small, with a poor dendritic growth, which were intermingled with some nestin positive fibers and blood vessels (Fig. 2). Compared with P16, P28 and P60, the somatic area at P9 was the smallest ($p < 0.01$, Fig. 3). From P11 to P16, the intensity of nestin immunohistochemical labeling, the somatic size and dendritic growth increased progressively, with a peak at P16 (Figs. 2–4). Subsequently, the staining intensity decreased accompanied with noticeable perikaryal shrinkage. Multiple comparison analysis revealed that the somatic area at P16 was significantly larger than those at P9, P28 and P60 ($p < 0.01$, Fig. 3), After P28, very little variation in the intensity of labeling, and also in the neuronal size or dendritic growth were noted. The neuronal labeling resembled adult configuration (Figs. 2 and 3). At P60, nestin positive neurons distributed exclusively in the MS-DBB. In the rostral part of BF, These neurons were arranged in a typical inversed V-shaped pattern. Positive cells in the medial septum were mainly medium-sized with elongated shape and oriented in parallel with the dorsoventral axis. Nestin-ir neurons in the diagonal band were relatively large and had a multipolar or oval shape oriented parallel with the main fiber bundles (data not shown). Between the P28 and P60, there were no significant differences of somatic area ($p > 0.05$, Fig. 3).

The results of the quantification of nestin immunoreactivity are showed in Fig. 5. A progressive increase in nestin positive counting was noted after P9. The nestin positive neurons within the DBB reached highest number at P16 and those within the MS reached highest number at P28.

In the P1-P11 cortex, striatum and subventricular zone (SVZ), nestin immunostaining revealed a dense pattern of radial fibers as well as numerous process-bearing astrocyte-like cells, which were intermingled with the radial fibers (Fig. 6). In P9-P12, Series of small varicosities appeared along the radial fibers, and drop-like cell-sized bodies were ‘hanging’ on the end of the radial fibers (Fig. 6). From P11, in the nestin immunostained sections radial fibers occurred scarcely (Fig. 6). At P16, as well as in adult, the nestin positive radial fibers almost completely disappeared. However, radial glia was rarely observed within the BF in the same stage. From P9 to P16, some nestin positive neuron-like cells were found existed in the lateral portion of the striatum. Most of these cells were round or oval in shape with large soma and one or two primary dendrites (Fig. 6). These nestin positive neuron-like cells reached highest number at P11 and disappeared at P16 (data not shown).

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