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Age-dependent Expression of GAP-43, Netrin-1, Collapsin-1, and Neuropilin-1 in Murine Cerebellum*

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Abstract GAP-43, netrin-1, collapsin-1, and neuropilin-1 have been regarded to play crucial roles in the formation of patterned neural connections. The cerebellum consists of five distinct concentric layers: white matter, internal granule layer (IGL), Purkinje cell layer (PCL), molecular layer (ML), and external granule layer (EGL) in young rodents. Cells in EGL are generated after birth. In contrast Purkinje neurons are born before birth, which receive main innervations of climbing fibers from the inferior olivary nucleus and parallel fibers from the internal granule cells. These innervations are mostly established in the first three postnatal weeks, accompanying the sprouting and maturation of Purkinje cells. The potential roles of GAP-43, netrin-1, collapsin-1 and neuropilin-1 in the postnatal development of cerebellum remain unclear. To get insights into the above issue, the expression of GAP-43, netrin-1, collapsin-1, and neuropilin-1 mRNAs and proteins were examined in the cerebellum of mice at postnatal days (P) 5, P10, P20 and adulthood. The results showed that these four molecules were expressed in different temporal and spatial patterns in the postnatal cerebellum of mice, which was in match with axonal synaptogenesis, elongation and synapse formation during postnatal development and adulthood. By using double immunohistochemistry, it was found that the Purkinje cells stained for GAP-43 were also positive for either netrin-1 or collapsin-1 at P10, and cells stained for collapsin-1 were also positive for netrin-1 or neuropilin-1. It was suggested that the four molecules are involved in the postnatal development of cerebellum.

Key words GAP-43, netrin-1, collapsin-1, neuropilin-1, neural plasticity, cerebellum, mouse
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Several groups of axon guidance molecules, i.e., GAP-43(axonal growth-associated protein 43), netrin-1, collapsin-1, and neuropilin-1 have been shown to have crucial roles in the formation of patterned neural connections. GAP-43 is a presynaptic protein, which is frequently used as a marker for sprouting, because its expression is located in growth cones [1]. Although mice lacking GAP-43 show no deficiency in nerve growth rate, and in extended neurites or growth cones of cultured neurons [2], GAP-43 is generally thought to require in navigating fibers to go past the midline decision point[3, 4], and to play a role in axonal synaptogenesis through action with G-proteins or calmodulin binding phosphoprotein [2~5]. Netrins are a conserved family of secreted proteins with some homology to laminin, including a series of three EGF-like repeats [6]. Besides the well known role for netrins i.e., attracting commissural axons, they also have roles in the guide of migrating cells and axonal growth cones [6]. Collapsins are secreted and transmembrane proteins, which significantly contribute to the maintenance and stability of neural networks [7]. They provide chemo-repulsive guidance [8~11], or chemo-attractive cues [11~15] for migrating axons, and appear to be involved in the cytoskeletal changes and endocytosis, leading to the collapse of growth cones[16, 17]. Neuropilin-1 is natural ligand for collapsins,

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as well as a co-receptor for vascular endothelial growth factor[11]. Collapsin-1 has been shown to repel the growing axons that express neuropilin-1[9, 18~22].

The cerebellum consists of five distinct concentric layers: white matter, internal granule layer (IGL), Purkinje cell layer (PCL), molecular layer (ML), and external granule layer (EGL) for young animals[23]. PCL, ML, and IGL contain Purkinje cells, interneurons, and granule cells, respectively, which are largely different in their morphologies and functions[23, 24]. Cells in EGL are generated after birth, although cell generation for Golgi interneurons and Purkinje neurons has been ceased by the day of birth[23, 25, 26]. Cells multiplying in the external germinal layer will definitely differentiate into basket cells during the second week, and most of the granule cells during the second and third week[27 ~30]. After postmitotic granule cells descend to the deep EGL, they become oriented perpendicular to the axons and attached to the Bergmann glia[25, 31]. Following radial migration through the molecular layer, they finally get to IGL, emitting long processes, the future parallel fibers[32~35].

Purkinje cells receive innervations of climbing fibers from the inferior olivary nucleus and parallel fibers from the internal granule cells. Some interneurons scattered in ML such as basket cells and stellate cells also innervate Purkinje cells. In rodents, these innervations are mostly established in the first three postnatal weeks, accompanying the sprouting and maturation of Purkinje cells. During the early postnatal stage of cerebellar development, both climbing fibers and parallel fibers are found to form transient synapses on the perisomatic processes and the outgrowing dendrite of Purkinje cells, respectively. Preceded by the development of the appropriate corticonuclear connectivity, as well as by an almost completely regular formation of the synaptic pattern of the cerebellar circuitry, some Purkinje cells are selectively lost during P15 and P45[36, 37]. Around 30 days after birth, the axonal terminals of climbing fibers and parallel fibers synapse on the dendrites of Purkinje cells[29].

Given the established roles for GAP-43, netrin-1, collapsin-1, and neuropilin-1, they most probably play roles in the development of cerebellum, especially in the establishment of neural connections upon Purkinje cells from different resources and in the competition between climbing fibers and parallel fibers. To our knowledge, only one or two of these axon guidance molecules were performed in previous reports, some of which are not always consistent. Thus, it is not yet clear that how these axon guidance related molecules are distributed in the postnatal cerebellum. Furthermore, it is not known whether these molecules were co-located in the same structures of cerebellum, due to the lack of double immunohistochemistry in previous reports. We thought that this issue is useful for understanding the roles which these axon guidance molecules play independently or cooperatively with other molecules in the postnatal development of cerebellum.

To address this issue, we first characterized the dynamical expression of these four molecules in the cerebellum of postnatal mice. By double immunohistochemistry, we then studied whether these molecules coexist in the same cerebellar cells or fibers. Our study may shed light on the functions of these four molecules during postnatal development and plasticity of cerebellum in mice.

1 Materials and methods

1.1 Animals and tissue preparation

Timed pregnant Kunming mice were bred from our own Laboratory at Beijing Normal University (Beijing, China), where they were checked daily for plugs. The plug date is considered embryonic day 1 (E1). The mice were kept under a 14 h/10 h light/dark cycle at 19～24℃. All experiment procedures were carried out in accordance with the guidelines of Beijing animal protection committee. The siblings were housed together in groups of two to six with their parents. The four age groups studied were postnatal 5, 10, and 20 days, and adulthood (>30 days) ($n = 4 \sim 6$ males for each age group). These age groups contained the periods of the development of the mouse cerebellum (P0 ～21). The mice were deeply anaesthetized by 20% ethyl carbamate (50 μl/g body weight). After perfusion with 0.9% saline and then with 4% cold paraformaldehyde in 0.1 mol/L phosphate-buffer (pH 7.4), the brains were extracted and postfixed 6 h in the same fixative at 4℃. Then the brains were immersed in 30% sucrose at 4℃ overnight and hemispheres from either side were cut into 10 μm thick sagittal sections on a freezing microtome (CM 1850, Leica).

1.2 *In situ* hybridization

Based on the sequence information available from

other species, we used PCR to amplify fragments of the GAP-43 cDNA (GenBank gi:64175448, full length of coding sequence, 748 bp, 97% similarity with nucleotides 1 to 717 of canary partial GAP-43 cDNA), netrin-1 (GenBank gi: 529418, 626 bp, nucleotides 528 to 1 153 of chicken netrin-1 cDNA), collapsin-1 cDNA (GenBank gi: 64175470, 425 bp, 94% similarity with nucleotides 1 290 to 1 714 of chicken semaphorin 3A cDNA) and neuropilin-1 (GenBank gi: 45382150, 484 bp, nucleotides 3 059 to 3 542 of chicken neuropilin-1 cDNA) from the mice. The total RNA was prepared from the brain of a P10 mouse by using the TRIZOL reagent (GIBCO). Reverse transcription was performed with M-MLV (Promega). For PCRs, primers were designed based on the sequence of the conserved region of GAP-43, netrin-1, collapsin-1 or neuropilin-1. The GAP-43 sense primer was 5′ CGAG ACAACCATGCTGTGCTGTATG 3′, and the anti-sense primer was 5′ TGGAAAGCCATT-TCTTAGAG TTCAG 3′. The netrin-1 sense primer was 5′ ACAACCTGACGTGCTGGCAGTCCG 3′, and the anti-sense primer was 5′ GTAGTGGAAGGG TT-TGCAGCGGTC 3′. The collapsin-1 sense primer was 5′ TATCCACGGCCAGGAA 3′, and the anti-sense primer was 5′ CGGTGTAAAGGAAGCTG 3′. The neuropilin-1 sense primer was 5′ GTGTTGAGA-CCAAGTATGATTGAC 3′, and the anti-sense primer was 5′ ACATA CACTAGCCAGT GCCAAGGA 3′. The yielded bands were cloned into pGEM-T Easy vector (Promega).

The sense and anti-sense cRNA probes were prepared by transcribing pGEM-T plasmid with T7 and SP6 RNA polymerase. *In vitro* transcription reactions were conducted according to the manufacturer's instructions for the digoxigenin (DIG) RNA labeling kit (Roche). Negative controls were prepared using the corresponding sense probes.

All the solutions used before hybridization step were RNase-free. Endogenous AP activity was quenched with 0.2 mol/L HCl followed by PBS rinses. Proteinase K (GIBCO) digestion was carried out at 37℃, followed by postfixation in 4% freshly depolymerized paraformaldehyde. The sections were acetylated with 0.25% (*v*/*v*) acetic anhydride (Sigma) in a 0.1 mol/L triethanolamine, pH 8.0 and 0.9% NaCl. Before hybridization, sections were incubated with hybridization solution (containing 50% deioned formamide, 5× SSC, 0.1 g/ml dextran sulfate, 5× Denhardt's solution and denatured salmon sperm DNA) at hybridization temperature (55℃ for GAP-43 anti-sense and sense probe, 52℃ for collapsin-1 anti-sense and sense probe; and 58℃ for the other anti-sense and sense probes) for 1 h. After incubation in hybridization solution containing RNA probes at appropriate concentration for 16 ～20 h, anti-DIG (1∶1 000) was dropped onto sections to react at room temperature for 2 h, and color-developed in the presence of nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Promega) for 48 h.

1.3 Immunohistochemistry

The sections were first incubated in 3% normal serum and then incubated with a primary antibody: mouse IgG monoclonal anti-GAP43 (Santa Cruz, 1∶300), rabbit IgG monoclonal anti-netrin-1 (Santa Cruz, 1∶300), goat IgG monoclonal anti-collapsin (Santa Cruz, 1∶300), or mouse IgG monoclonal anti-neuropilin-1 (Santa Cruz, 1∶300). Before use, these primary antibodies have been confirmed to be able to react with mice specifically by Western blotting. The secondary antibody were biotinylated horse anti-mouse IgG or rabbit biotinylated anti-goat IgG (Santa Cruz, 1∶200), or biotinylated goat anti-rabbit IgG (Jackson, 1∶400), followed by an avidinbiotin-peroxidase complex (ABC; Vector Laboratories, 1∶150). Color development was achieved by incubating with 3, 3′-diaminobenzidine 4-HCl (DAB; Sigma). Each step was followed by PBS washing. In the control experiments, the primary or secondary antiserum was omitted from the immunohistochemical protocol. Generally, the sections at different ages were performed in the same staining experiment.

For double immunohistochemistry, after the sections were treated with the primary antibodies as described above, the sections were incubated with the secondary antibody: Rhodamine-conjugated donkey anti-mouse IgG (Jackson, 1∶200), FITC-conjugated donkey anti-rabbit IgG (Jackson, 1∶200), FITC-conjugated donkey anti-mouse IgG (Jackson, 1∶200), FITC-conjugated donkey anti-goat IgG (Jackson, 1∶200), or Cy3-conjugated donkey anti-rabbit IgG (Jackson, 1∶200).

1.4 Photography and construction of multicolored epifluorescent images

The images of targeted areas were obtained with CoolSNAP color digital camera (Photometrics) attached to an Olympus microscope (BH-2).

Fluorescence signals were detected with an Olympus fluorescent microscope and spot digital camera (Diagnostic Instruments) at excitation/emission wavelength of 492 nm/520 nm (FITC, green) and 550 nm/570 nm (Rhodamine or Cy3, red). The images were corrected for brightness and contrasted by Adobe Photoshop 8.0 (Adobe Systems).

2 Results

2.1 *In situ* hybridization and immunohistochemistry

2.1.1 GAP-43.

At P5, strong labeling for *GAP-43* mRNA appeared in EGL (Figure 1a and b). Positive staining was located in a superficial area just beneath the pia, with about five layers of labeled cells wide (Figure 1a and b). At this age, labeled cells in IGL were not as densely distributed as those in EGL (Figure 1a), and ML is still not well developed[27~30]. At P10, *GAP-43* mRNA nearly stopped expression in EGL (Figure 1c and d). ML could be distinguished at P10, but it was lack of *GAP-43* mRNA expression (Figure 1c and d). Purkinje cells and cells in IGL showed strong expression of *GAP-43* mRNA at P10 (Figure 1c and d). However, Purkinje cells were not easily distinguished from those in IGL at this period (Figure 1c and d). At P20, EGL disappeared, and *GAP-43* mRNA expression was moderate in PL, and light in ML and IGL (Figure 1e). At adulthood, *GAP-43* mRNA expression became strong in PL and IGL, and remained weak in ML (Figure 1f and g).

For GAP-43 protein, some labeled fibers in EGL and cells in IGL were seen at P5 (Figure 1h). At P10, dense labeling appeared in ML, and moderate or weak labeling occurred in EGL and IGL (Figure 1i). At P20, ML was filled with the extending dendrites of Purkinje cells positive for GAP-43 protein, but the perikarya of Purkinje cells were not positively stained (Figure 1j). At adulthood, immunohistochemistry for GAP-43 protein showed similar pattern to that at P20. ML was filled with labeled parallel fibers, extending from PL to the cerebellar surface (Figure 1k and l). At P20 and adult, plenty of varicosities were found to locate along with GAP-43 fibers (Figure 1j～l).

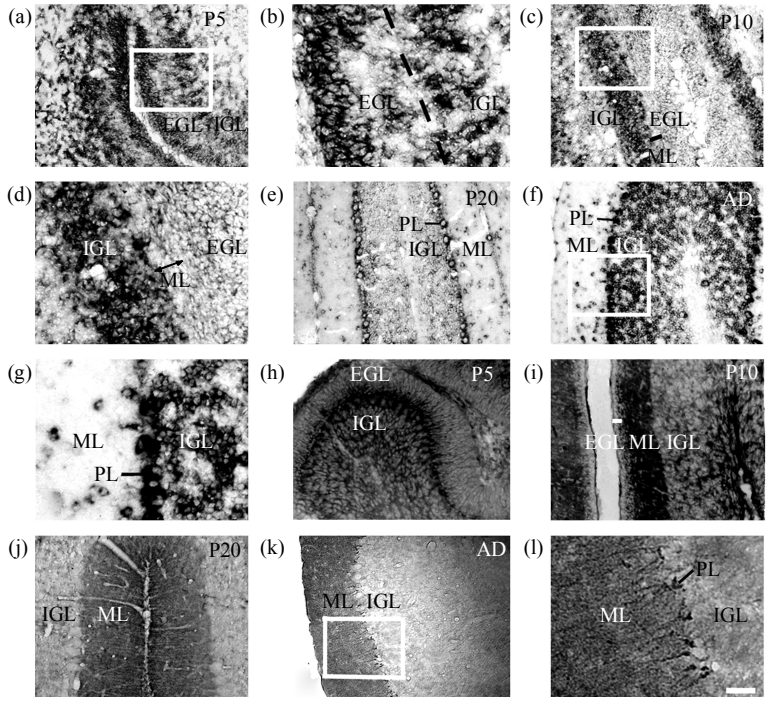

Fig. 1　GAP-43 mRNA (a～f) and protein (g～l) in the sagittal sections of the cerebellum in postnatal mice

(a) P5, labeling was detected in EGL and IGL. (b) High-power photomicrographs of GAP-43 expression in (a). (c, d) Low expression was detected in EGL and strong or moderate expression was detected in IGL at P10. (e) Cell staining in PL and low expression in ML and IGL at P20. (f, g) Expression at adulthood. (h, l) Expression of GAP-43 protein at P5 (h), P10 (i), P20 (j) and adulthood (k, l). Boxed areas in (a), (c), (f), and (k) were magnified in (b), (d), (g) and (l), respectively. EGL: External granular layer, ML: Molecular layer, IGL: Internal granular layer, PL: Purkinje layer, AD: Adult. Scale bar: (a), (c), (e), (f), (h), (i), (j), (k), 60 μm; (b), (d), (g) and (l), 38 μm.

2.1.2 Netrin-1.

Moderate labeling for *netrin-1* mRNA appeared in EGL and IGL at P5 (Figure 2a and b). The expression of *netrin-1* mRNA largely decreased in EGL, but still remained moderate in IGL at P10 (Figure 2c). From P20 onward, labeling was similar between different age groups, and it was mainly confined to PL (Figure 2d and e).

For netrin-1 protein, Purkinje cells were positively labeled at all the studied age groups, and labeled fibers were mainly located in ML(Figure 2f～l). Although Purkinje cells were stained at all the age groups, they exhibited different characteristics. At P5, Purkinje cells were aligned to a monolayer, sending short primary dendrites from their bodies (Figure 2f). At P10, Purkinje cells were more intensely stained, and longer dendrites were extended, in comparison with those at P5 (Figure 2g and h). At P20, Purkinje cells were characterized by the extended processes from perikarya, generally vertical to the pial surface, and parallel to each other (Figure 2i and j). From P20 onward, Purkinje cells were moderately stained, and radial dendrites were weakly stained (Figure 2g and h).

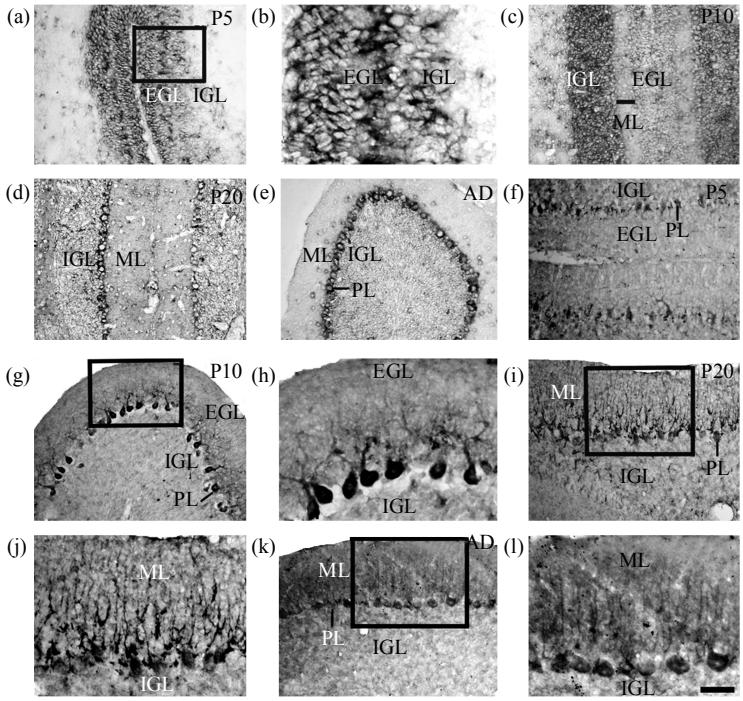

Fig. 2 Netrin-1 mRNA (a～e) and protein (f～l) in the sagittal sections of the cerebellum in postnatal mice

(a, b) netrin-1 mRNA expression at P5 is prominent in cells of EGL and IGL. (c) Expression is moderate in IGL and low or absent in ML and EGL at P10. (d, e) Expression at P20 (d) and AD (e). Expression of netrin-1 protein at P5 (f), P10 (g, h), P20 (i, j) and adulthood (k, l). Boxed areas in (a), (g), (i) and (k) were magnified in (b), (h), (j) and (l), respectively. EGL: External granular layer; ML: Molecular layer; IGL: Internal granular layer; PL: Purkinje layer; AD: Adult. Scale bar: (a), (c), (d), (e), (f), (g), (i), (k), 60 μm; (b), 38 μm; (h), 35 μm; (j) and (l), 30 μm.

2.1.3 Collapsin-1.

Strong or moderate expression for *collapsin-1* mRNA was observed in EGL and IGL at P5 (Figure 3a and b). From P10 to adulthood, Purkinje cells were heavily or moderately labeled with collapsin-1 mRNA (Figure 3c～f). In contrast, weak or moderate staining was seen in EGL, IGL and ML (Figure 3c～f).

Labeling for *collapsin-1* protein was largely consistent with that *in situ* hybridization. Perikarya of Purkinje cells, as well as the processes extended from cell bodies were positively stained with collapsin-1 protein from P5 onwards. No labeled cells were seen in the other cerebellar regions, except IGL at P5 and adulthood, and ML at adulthood (Figure 3g～l). At P20, the labeled parallel dendrites were filled with ML, vertical to the cerebellar surface (Figure 3i and j).

2.1.4 Neuropilin-1.

For *neuropilin-1* mRNA, positive staining occurred in EGL at P5 and P10(Figure 4a～c). At P20, some labeled small round cells were seen in ML, and positive labeling remained in PL and IGL (Figure 4d). At adulthood, only PL and ML were weakly stained

(Figure 4e).

For immunohistochemistry of neuropilin-1, positive labeling was seen in the growing neurites and perikarya of Purkinje cells at P5 and P10(Figure 4f～h). At P20, labeled dendrites were seen to extend to the surface of cerebellum, parallel to each other, and vertical to the cerebellar surface (Figure 4i and j). At adulthood, only the bodies of Purkinje cells were stained with neuropilin-1 (Figure 4k and l). Some cells labeled for neuropilin-1 were scattered in ML at adulthood, and few ones were seen in IGL (Figure 4k and l).

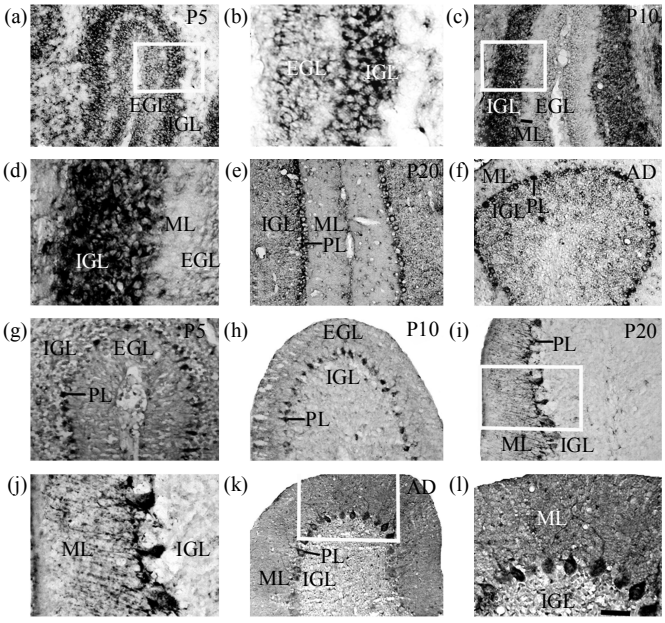

Fig. 3 Collapsin-1 mRNA (a～f) and protein (g～l) in the sagittal sections of the cerebellum in postnatal mice

(a, b) Collapsin-1 mRNA expression at P5 is prominent in cells of EGL and IGL. (c, d) Expression in IGL at P10. (e, f) Expression at P20 (e) and AD (f). Expression of collapsin-1 protein at P5 (g), P10 (h), P20 (i, j) and adulthood (k, l). Boxed areas in (a), (c), (i) and (k) were magnified in (b), (d), (j) and (l), respectively. EGL: External granular layer; ML: Molecular layer; IGL: Internal granular layer; PL: Purkinje layer; AD: Adult. Scale bar: (a), (c), (e), (f), (g), (h), (i), (k), 60 μm; (b), and (d), 38 μm; (j) and (l), 30 μm.

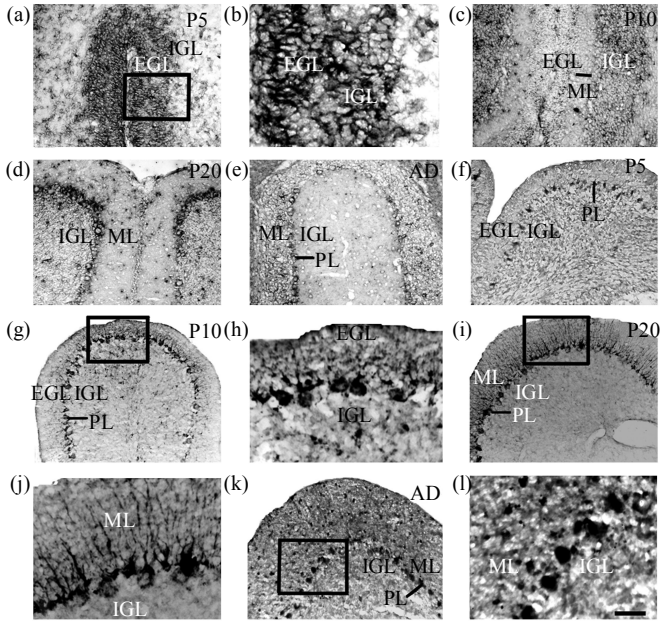

Fig. 4 Neuropilin-1 mRNA (a～e) and protein (f～l) in the sagittal sections of the cerebellum in postnatal mice

(a, b) Early postnatal neuropilin-1 hybridization is confined to EGL and IGL at P5. (c) Low expression in EGL, ML and IGL at P10. (d, e) Expression at P20 (d) and AD (e). Expression of neuropulin-1 protein at P5 (f), P10 (g, h), P20 (i, j) and adulthood (k, l). Boxed areas in (a), (g), (i), and (k) were magnified in (b), (h), (j) and (l), respectively. EGL: External granular layer, ML: Molecular layer; IGL: Internal granular layer; PL: Purkinje layer; AD: Adult. Scale bar: (a), (c), (d), (e), (f), (g), (i), (k), 60 μm; (b) and (h), 38 μm; (j) and (l), 30 μm.

2.2 Double immunohistochemistry

To know whether GAP-43, netrin-1, collapsin-1 and neuropilin-1 coexist in the same Purkinje cells or their fibers, we performed double immunohistochemistry at P10. This stage was chosen for both the cell bodies and fibers of Purkinje cells were positively labeled for all the four molecules. As shown in Figure 5a and b, some double-labeled cells for GAP-43 and netrin-1 or collapsin-1 could be seen. However, since ML was heavily stained for GAP-43, the bodies and fibers of Purkinje cells were not easily distinguished individually. The double immunochemistry for GAP-43 and neuropilin-1 was not able to be performed, for the bodies for the two substances were both raised in mice. Many double-labeled neuropilin-1/collapsin-1 (Figure 5c) and netrin-1/collapsin-1 (Figure 5d) Purkinje cells were observed. These cells were easily recognized by their location, large size, and the labeled proximal part of the apical and some horizontally oriented dendrites.

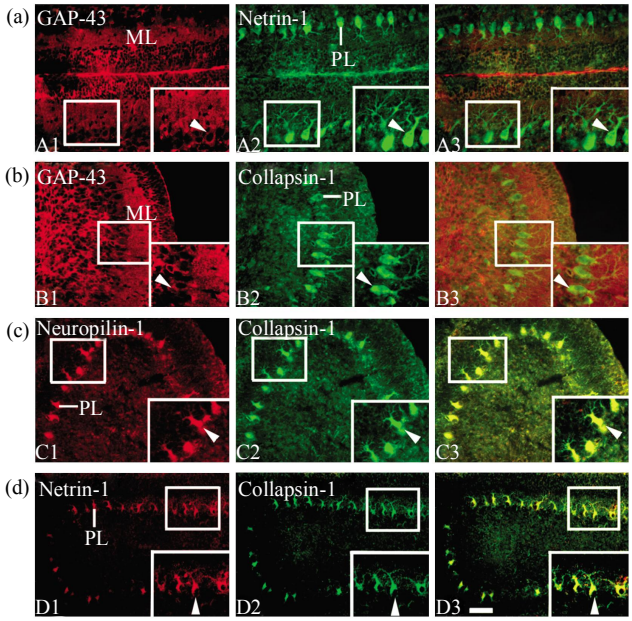

Fig. 5 Double immunohistochemistry for GAP-43, netrin-1, collapsin-1 and neuropilin-1 in the sagittal sections of the cerebellum at P10

(a) Double labeling for GAP-43 and netrin-1. (b) Double labeling for GAP-43 and collapsin-1. (c) Double labeling for neuropilin-1 and collapsin-1. (d) Double labeling for netrin-1 and collapsin-1. Insets in A1～D3 are the magnifications of the boxed areas, respectively. Arrowheads in insets of A1～D3 indicate double-labeled cells. ML: Molecular layer; PL: Purkinje layer. Scale bar: A1～C3, 50 μm; D1～D3, 70 μm; insets in A1～C3, 20 μm; insets in D1～D3, 30 μm.

3 Discussion

3.1 Postnatal expression pattern of GAP-43 in the cerebellum

Our study showed that intense labeling for *GAP-43* mRNA appeared in the outer part of EGL at P5, then moved towards ML, and disappeared after P20. Our study also indicated that persistent moderate expression of *GAP-43* mRNA occurred in IGL from P5 to adulthood, consistent with two previous studies[38, 39]. According to our study, labeling was seen in Purkinje cells from P5 onward. Although the above result was only coincided with one report[37], and not with others[39～41], it was confirmed by our immunohistochemistry study.

As shown above, expression of GAP-43 protein was largely consistent with that of *GAP-43* mRNA. The dendrites of Purkinje cells began to extend towards ML at P5, and entered ML at P10. After P20, the extended parallel dendrites reached to the pial surface of cerebellum. These results agree with previous reports on the basis of autoradiographic, histological (including Golgi), histochemical, and electron microscopic techniques. According to these studies, the maturation of Purkinje cells includes several major events. First, Purkinje cells whose

synapses are yet formed are dispersed into a monolayer in the first postnatal week. Second, the planar arbors of Purkinje cells fully develop in the molecular layer after the second postnatal week [29, 42]. Third, the dendritic spines of Purkinje cells begin to synapse with the parallel fiber in ML during the second week after birth. Finally, perisomatic synapses of Purkinje cells with climbing fibers were formed around one week after birth, but they disappeared before the soma of Purkinje cells is invaded by permanent inconspicuous synapses of basket cells during the second week. Permanent synapses in the dendrites of Purkinje cells with climbing fibers are established after the second postnatal week, and peaked around the third postnatal week. As shown in our results, plenty of varicosities were exclusively found to locate along the dendrites of Purkinje cells at adulthood. On the basis of the above reports, these varicosities most probably indicate the forming synapses in the dendrites of Purkinje cells with parallel fibers, climbing fibers or/and fibers from interneurons. These results indicate that GAP-43 is probably involved in axonal synaptogenesis, elongation, nerve sprouting and synapse formation during development and at adulthood [5 ~ 7]. This is consistent with the established notion that GAP-43 plays a role in synaptic plasticity, participating in membrane extension during neuritogenesis, in neurotransmitter release and long-term potentiation[1].

3.2 Postnatal expression pattern of netrin-1 in the cerebellum

Our study indicated that cells for mRNA and protein of netrin-1 were mainly distributed in PL, and only some cells were sparsely located in ML and IGL. It has been shown that netrin RNA is strongly expressed in the developing spinal cord, and the ventral ventricular zone in the mouse and chicken[43 ~ 46], and netrin-1 guides the migration of precerebellar, cerebellar, and hypothalamic neurons[47 ~ 51]. In addition, Ryder and Cepko[52] mimic the tangential migration of granule cells within the deeper EGL, and found that the tangential migration was under the control of *netrin-1*. Our study to show that netrin-1 was expressed in EGL and ML suggests that netrin-1 may be involved in the migration of granule cells, and netrin-1 may contribute to the initiation of the correct parallel fiber extension, a prerequisite for the ordered exit of postmitotic premigratory granule cells from the EGL [53]. Our double immunohistochemistry showed that netrin-1 was co-located with collapsin-1 in some cerebellum cells at studied age groups.

It has been shown that Netrin-1 plays several roles in the development of nervous system. (1) Netrin-1 is the prototypical axon guidance cue to orient many neurons or axons, and it exerts a strong chemoattractive effect on migrating neurons and promotes the exit of postmitotic migrating neurons from their birthplaces [54]. (2) Netrin-1 is a long-range diffusible factor that exerts chemoattractive or chemorepulsive effects on developing axons growing to or away from the neural midline[55]. Netrin-1 exerts a strong chemoattractive effect on migrating neurons and promotes the exit of postmitotic migrating neurons from the embryonic sources [55]. (3) In the postnatal cerebellum, netrin-1 repels both the parallel fibres and migrating granule cells growing out from explants taken from the external germinal layer[55]. Considering these reports, our results suggest that netrin-1 may be involved in the development of cerebellum, including the formation of the synapses of Purkinje cells with parallel fibers, climbing fibers or/and fibers from interneurons.

3.3 Postnatal expression pattern of collapsin-1 and neuopilin-1 in the cerebellum

As shown in the present study, both mRNA and protein of collapsin-1 or neuropilin-1 were located in Purkinje cells in all studied age groups, while they only occurred in ML and IGL at some age groups. Our report was largely consistent with previous reports on Purkinje cells[11, 15, 56 ~ 59]. To date, there is only one report to show that collapsin-1 is located in the IGL of adult schizophrenic human[60].

Collapsin-1 and neuropilin-1 were both strongly expressed in the dendrites of Purkinje cells at P10 and P20. This suggests that both collapsin-1 and neuropilin-1 are involved in forming the synapses of Purkinje cells with parallel fibers, climbing fibers or/and fibers from interneurons such as basket cells and stellate cells in ML. Collapsin-1 might be essential for the orderly arrangement of Purkinje cells occupied by this protein.

Our double immunohistochemistry showed that collapsin-1 and neuropilin-1 have the same distribution in the P10 cerebellum. This is reasonable considering that neuropilin-1 is a receptor of collapsin-1, and co-participation of neuropilin receptors is required for collapsin-1 to exert a chemorepulsive response. It is generally known that collapsin-1 provides chemo-repulsive guidance [8 ~ 11], or chemoattractive cues [11 ~ 15]

for migrating axons. The presence of collapsin-1 and neuropilin-1 in the dendrites of Purkinje cells at P10 and P20 suggests that chemo-repulsive or attractive guidance and the collapse of growth cones most probably occur during the formation of the synapses of Purkinje cells with parallel fibers, climbing fibers or/and fibers from interneurons, and that collapsin-1 and neuropilin-1 are involved in the competition between climbing fibers and parallel fibers synapsing on Purkinje cells [29]. In addition, some labeling for collapsin-1 and neuropilin-1 occurred in Purkinje cell bodies or their surroundings at adulthood supports the notion that the synaptic plasticity of cerebellum still happen at adults, obtained from GAP-43 study. It is possible that collapsin-1, neuropilin-1 are concerned with the long-term modifications of parallel fiber-Purkinje cell synapse and the change from multi-one model to one-one model of climbing fiber-Purkinje cell connection during development [61] or at physiological conditions[62].

Our study is largely consistent with previous reports on the roles of collapsin-1 and neuropilin-1 in the development of brain. According to the study of Shirvan *et al*. [63], collapsin-1 is suggested to play a major role in navigating axonal networks throughout development into their correct destinations. In addition, it has been shown that the radial migration of rat layer Ⅱ/Ⅲ cortical neurons requires guidance by the extracellular diffusible factor Sema3A, which is expressed in a descending gradient across the cortical layers, whereas its receptor neuropilin-1 is highly expressed in migrating neurons [64]. Downregulation or conditional knockout of neuropilin-1 in newborn cortical neurons impedes their radial migration by disrupting their radial orientation during migration without altering their cell fate[64].

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GAP-43，Netrin-1，Collapsin-1 和 Neuropilin-1 在出生后小鼠小脑中的动态表达 *

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摘要 GAP-43，netrin-1，collapsin-1 和 neuropilin-1 被认为在成网络分布的神经联系中发挥重要的作用．在年幼的啮齿类动物中，小脑包含 5 种不同的集中分布层：白质、内颗粒细胞层(IGL)、浦肯野氏细胞层(PCL)、分子层(ML)和外颗粒细胞层(EGL)．与浦肯野氏神经元在出生前产生这一点不同的是，EGL 中的细胞在出生后产生，它们接受从前脑 olivary 核团发出的攀援纤维的主要神经投射，以及从内颗粒细胞发出的平行纤维的神经投射．这些神经投射主要在出生后的前 3 个星期内建立，同时还有浦肯野氏细胞的发育和成熟．而 GAP-43，netrin-1，collapsin-1 和 neuropilin-1 在出生后小脑发育的潜在作用仍然不清楚．为了更加清楚地探讨上述问题，检验了 GAP-43，netrin-1，collapsin-1 和 neuropilin-1 的 mRNA 与蛋白质在出生后 5，10，20 天和成年小鼠小脑中的表达情况．研究结果显示，这 4 种分子在小鼠出生后的小脑中有不同的时间和空间表达形式，这些结果与出生后发育和成年期间的轴突发生、延伸以及突触形成都有关联．通过免疫组织化学双标染色，发现小鼠出生后 10 天的小脑中，GAP-43 阳性的浦肯野氏细胞也显示 netrin-1 或 collapsin-1 阳性，并且 collapsin-1 阳性的细胞也对 netrin-1 阳性．上述研究结果证明这 4 种分子可能参与了小脑的出生后发育．

关键词 GAP-43，netrin-1，collapsin-1，neuropilin-1，神经可塑性，小脑，小鼠

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