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Research report

Developmental expression of γ-aminobutyric acid transporters (GAT-1 and GAT-3) in the rat cerebellum: evidence for a transient presence of GAT-1 in Purkinje cells

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Abstract

The cerebellar cortex contains several classes of GABAergic neurons. Previous studies have shown that most GABAergic neurons in this region possess the capacity for γ-aminobutyric acid (GABA) uptake. The present study determined the postnatal expression of two GABA transporters, GAT-1 and GAT-3, in the cerebellar cortex and deep nuclei of the rat by using immunocytochemistry. Immunoreactivity for GAT-1 and GAT-3 appears at postnatal day 7 (P7), emerges centroperipherally across the cerebellum during the following 2 weeks and reaches an adult-like pattern by P30. The mature patterns are fully established by P45, which for GAT-1 is characterized by immunolabeled profiles localized exclusively to neuropil, mostly in the molecular layer and the pinceaux deep to the Purkinje cell bodies, and for GAT-3 as immunoreactivity distributed in the neuropil of mainly the granular layer. Before the adult patterns are completed, GAT-1 immunoreactivity is present in the somata of Purkinje, Golgi, basket and stellate cells between P7 and P21, while GAT-3 immunoreactivity is distinct in astrocytic somata which are organized in regularly spaced clusters. During this period, there is also a banding pattern in the sagittal plane of GAT-1 immunoreactivity in developing Purkinje cells. The postnatal development of GAT-1 and GAT-3 in the rat cerebellar cortex shares a similar spatiotemporal pattern with other GABAergic parameters, including the GABA synthesizing enzyme, GABA content and uptake. Specifically, the transient expression of GAT-1 in the somata and dendrites of cerebellar GABAergic neurons appears to correlate with the supra-adult levels of whole-tissue GABA uptake capability during development. Further, GAT-1 expression in immature Purkinje cells may play a unique role in regulating GABA's function during development, since mature Purkinje cells do not express GAT-1 or take up GABA.

Keywords: Purkinje cell; Interneuron; Astrocyte; Dentate nucleus; Zonal organization

1. Introduction

The mammalian cerebellar cortex contains at least four types of neurons that utilize γ-aminobutyric acid (GABA) as a neurotransmitter. They are the basket and stellate cells in the molecular layer, the Purkinje cells in the Purkinje cell layer and the Golgi cells in the granular layer [8,16]. The GABAergic identification of these neurons has been established by autoradiographic localization of [^{3}H]GABA accumulation [7,27,34,58,59]; by electrophysiological detection of inhibitory responses in postsynaptic neurons following iontophoretical application of GABA [47]; and by direct visualization of GABA or its synthesizing enzyme by means of immunocytochemistry and in situ hybridization [9,17,18,20,48,56,57,64]. It should be noted that the mature cerebellar Purkinje cells do not take up [^{3}H]GABA when it is placed into the cerebellar cortex, deep cerebellar nuclei or vestibular nuclei, whereas all other GABAergic neurons accumulate GABA in vivo and in vitro [7,27,34,58,59,62]. Thus, it appears that the Purkinje cells lack an effective GABA uptake system.

GABA uptake is mediated by its high affinity transport system. The latter belongs to a GABA transporter (GAT) family consisting of translemmal proteins expressed in the nervous system and peripheral tissue [13,14,31,33,37]. To date, four GATs have been cloned and they are classified as GAT-1, GAT-2, GAT-3 and GAT-4 according to their differential amino acid sequences and pharmacological properties [5,11,15,22,37–39,53]. GABA uptake by GAT-1 is strongly inhibited by *cis*-3-aminocyclohexane carboxylic

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acid (ACHC) but not by β-alanine. GAT-2 and GAT-3 mediated GABA uptake is inhibited by β-alanine but not by ACHC. GABA uptake by GAT-4 is inhibited by neither agent [11,31,38]. The cellular localization of GATs in the brain has been recently described, and it has been shown that GAT-1 and GAT-3 are the major GATs in the central nervous system [15,29,43,44,52–55,61,71]. Generally, GAT-1 is expressed in both neurons and astrocytes, whereas GAT-3 appears only in the latter. However, areal differences in GAT-1 expression exist in neurons across the mature central nervous system. For example, in the cerebral cortex GAT-1 mRNA is expressed in the somata of GABAergic interneurons, whereas GAT-1 immunoreactivity is mainly localized to presynaptic terminals of these neurons [43,44,52,53,55,61,66]. In contrast, in the retina both GAT-1 mRNA and immunoreactivity are present in the somata of amacrine cells (retinal ganglion cells also express GAT-3) [6,28,32,72]. In the mature cerebellar cortex, the basket, stellate and Golgi cells express GAT-1 mRNA signal in their somata and GAT-1 immunoreactivity in their axon terminals. However, for the Purkinje cell, not only is GAT-1 mRNA undetectable in its cell body, but also GAT-1 immunoreactivity is absent in its axon terminals [15,29,30,45,52–54]. The lack of GAT-1 mRNA and protein in the Purkinje cells is consistent with the finding that Purkinje cells do not take up GABA in the adult.

We recently revealed a novel finding that the somata of neocortical and hippocampal interneurons which display little GAT-1 immunoreactivity in the adult exhibit a transient immunostaining during development [66]. As the cerebellar cortex contains multiple types of GABAergic neurons with a more variable GABA uptake ability in the adult, it is of great interest to determine how GABA transporters are developmentally expressed in this brain region. Therefore, in the present study the postnatal expression of GAT-1 and GAT-3 in the rat cerebellum was examined by immunocytochemical methods.

2. Materials and methods

2.1. Animals

Male and female albino rats (Sprague–Dawley) at different postnatal ages were used. The day of birth was defined as postnatal day 0 (P0) and the ages of the rats studied in the present investigation were P0, P5, P7, P10, P14, P21, P30, P45 and P60. Three or four animals were examined from each of these times.

2.2. Tissue preparation

The animals were deeply anesthetized with i.p. injections of sodium pentobarbital (50 mg/kg) before transcardiac perfusion with 0.1 M phosphate buffered saline (PBS, pH 7.4), followed by 4% paraformaldehyde and 0.5% glutaraldehyde in phosphate buffer (pH 7.4). The brains were removed and postfixed by placing them in the perfusion solution for 4–8 h at 4°C. The tissue was then transferred to cold PBS for several hours to a few days. The cerebellum and brainstem were sectioned in the coronal plane at 50 μm with a vibratome. Alternate sections through the rostrocaudal span of the cerebellum were collected in PBS and processed for immunocytochemistry and Nissl staining (Cresyl violet).

2.3. Immunocytochemistry

Free-floating sections were immunostained for GAT-1 and GAT-3 by the standard avidin–biotin complex (ABC) method. Briefly, endogenous peroxidase activity was eliminated with a 20-min incubation in 0.1% H_2O_2. Background staining was diminished by a 2-h incubation in 5% normal goat serum (NGS) at room temperature, after which the sections were incubated in a PBS solution containing 5% NGS, 0.3% Triton X-100 and rabbit anti-GAT-1 or GAT-3 primary antibodies at a final dilution of 1:5000 overnight at 4°C and with agitation. The tissue was further incubated in 1% goat anti-rabbit IgG with 5% NGS in PBS for 1 h at room temperature, followed by an incubation in 1% ABC solution for another hour. The bound antibodies were finally visualized with 0.05% diaminobenzidine and 0.005% H_2O_2. Three 10-min rinses with PBS were used between all incubations.

The primary antibodies were kindly provided by Dr. N.C. Brecha at the University of California at Los Angeles. The specificity of these antibodies was verified in previous studies [43,44]. Other immunoreactive reagents were obtained from Vector Laboratories. In this study, processing the sections with the primary antibody omitted, or replaced by normal rabbit serum, yielded no specific immunolabeling. In several experiments, brain sections from two or more age groups were processed at the same time under identical conditions.

3. Results

3.1. Immunoreactivity at P0–P7

Immunoreactivity for GAT-1 was first detected in the developing cerebellar cortex at P7 (Fig. 1A), and was mainly found in 2–4 central vermal folia, where it appeared in the differentiating Purkinje cell and molecular layers. The immunolabeled perikarya resembled Purkinje cell precursors. In other vermal folia located rostral, caudal, dorsal and ventral to the aforementioned central ones, however, GAT-1 immunolabeling was light and only found in the neuropil of the middle part of the cortex. Furthermore, GAT-1 immunoreactivity was very faint or not

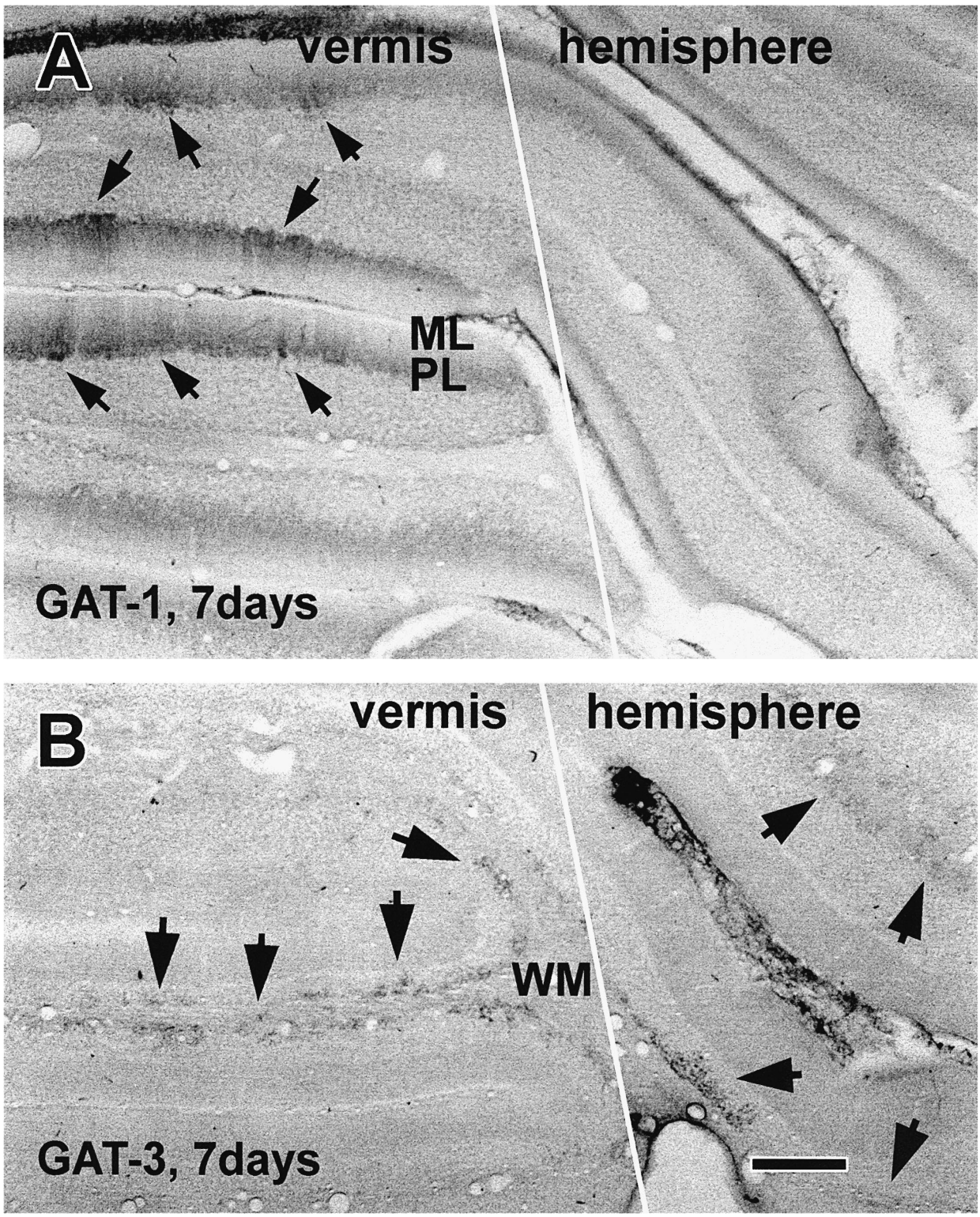

Fig. 1. Distribution of GAT-1 (A) and GAT-3 (B) immunoreactivities in the cerebellar cortex of a 7 day-old rat. (A) Shows GAT-1 immunolabeling (arrows) at the middle portion of the cortex of two adjacent vermal lobules (III and IV), corresponding to the developing Purkinje cell layer (PL) and inner molecular layer (ML). (B) Shows GAT-3 immunolabeling that appears as separate profiles (arrows) at the border of the cortex and the white matter (WM). For both (A) and (B) the vermis is separated from the hemispheres by a white line. Scale bar = 500 μm.

detected in the cerebellar hemispheres at this age. Thus, GAT-1 immunoreactivity displayed a centroperipheral gradient across the cerebellar cortex (Fig. 1A).

GAT-3 immunoreactivity at P7 appeared as individual small profiles distributed at the border between the cortex and the white matter (Fig. 1B), and was mainly found in

the vermis and the paravermal portion of the hemispheric lobules. The immunoreactive elements were arranged in clusters, that were later identified as glial cells (see below).

3.2. Immunoreactivity at P10

At P10, GAT-1 immunoreactivity was found in numerous somata in most vermal folia (Fig. 2A). In contrast to the Purkinje cells in the vermis, those in the hemispheres remained unlabeled at this age, though faint neuropil labeling now appeared in the differentiating Purkinje cell and molecular layers. Most immunolabeled somal profiles in the vermis were clearly identified as the cell bodies and dendrites of immature Purkinje cells. They were organized in a cellular layer consisting of one to two rows of cells at the middle of the cortex in cross-sectioned folia (Fig. 2B). In addition to the centroperipheral gradient (Fig. 2A), GAT-1 immunoreactivity in the somata and dendrites of Purkinje cells also showed a banding pattern, which was most distinct in the folia that were adjacent to the ones

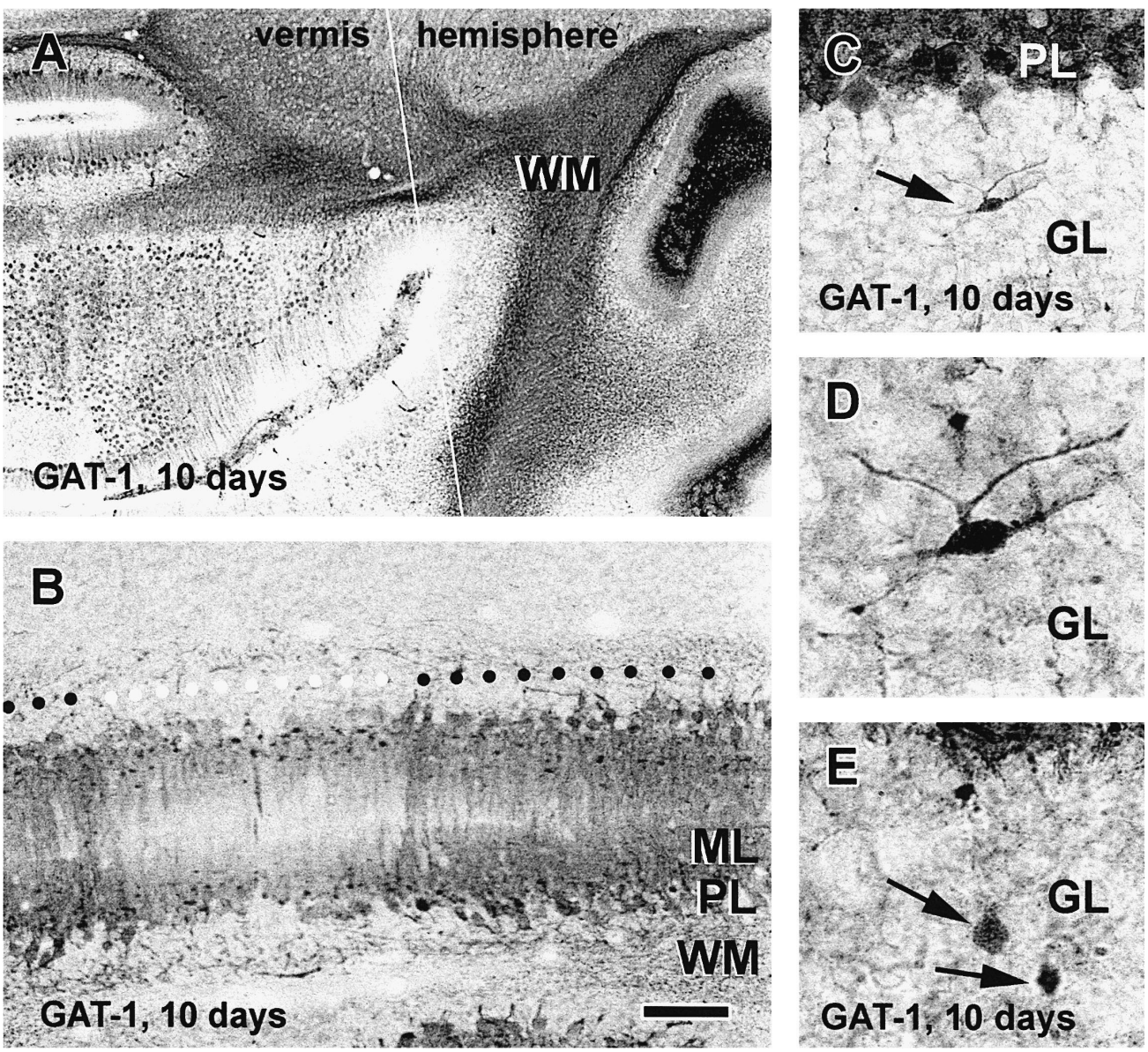

Fig. 2. Distribution and morphology of GAT-1 immunoreactive profiles in the rat cerebellar cortex at postnatal day 10 (P10). (A) Shows numerous immunolabeled somata that appear in several vermal lobules but none are found in the hemispheres. Note the labeling found in the white matter (WM). (B) Is an enlargement of the labeled cells in the Purkinje cell layer (PL) with their labeled dendrites projecting into the molecular layer (ML) and axons in the WM. Note that the immunoreactivity in the Purkinje cells of the vermal cortex is not uniform along the long axis of the cerebellar folia. Regions with intensely labeled Purkinje cells (indicated with black dots) alternate with zones occupied by Purkinje cells that are either lightly immunostained or not labeled (indicated with white dots). Lightly-immunostained small cell bodies are found in the ML (B). (C–E) Show small to medium-sized neuronal somata (arrows) immunoreactive for GAT-1 in the granular layer (GL). The labeled soma and dendrites of the cell in C (arrow) are enlarged in D. Scale bar = 500 μm for (A), 200 μm for (B), 100 μm for (C) and 20 μm for (D, E).

where the Purkinje cells were not yet immunolabeled for GAT-1 (Fig. 2B, also see Fig. 4B). The bands consisted of zones with intensely stained Purkinje cell bodies and dendrites interposed with those containing lightly stained or unlabeled cells (Fig. 2B). The axons of Purkinje cells were also immunolabeled for GAT-1, and thus, there was moderate labeling in the white matter (Fig. 2A,B; Section 3.6). In addition to the Purkinje cells, some smaller perikarya became immunolabeled for GAT-1 at this stage, and were mostly located in the vermal cortex. Those in the molecular layer were lightly labeled, and were especially distinct in the bands lacking immunolabeled Purkinje cells (Fig. 2B). Other small to medium-sized GAT-1 immunoreactive somata were infrequently observed in the granular layer (Fig. 2C–E). They resembled Golgi cells in their distribution and dendritic arbors [50].

At P10, GAT-3 immunoreactive profiles were more numerous and distinct compared to those at P7 (Fig. 3A,B). They were found in all vermal lobules and most hemispheric lobules, and remained distributed at the cortex–white matter border. At higher magnification, GAT-3 immunostaining occurred in spider-like cells consisting of very small somata with multiple and extensively branched processes (Fig. 3C). The morphological features of these

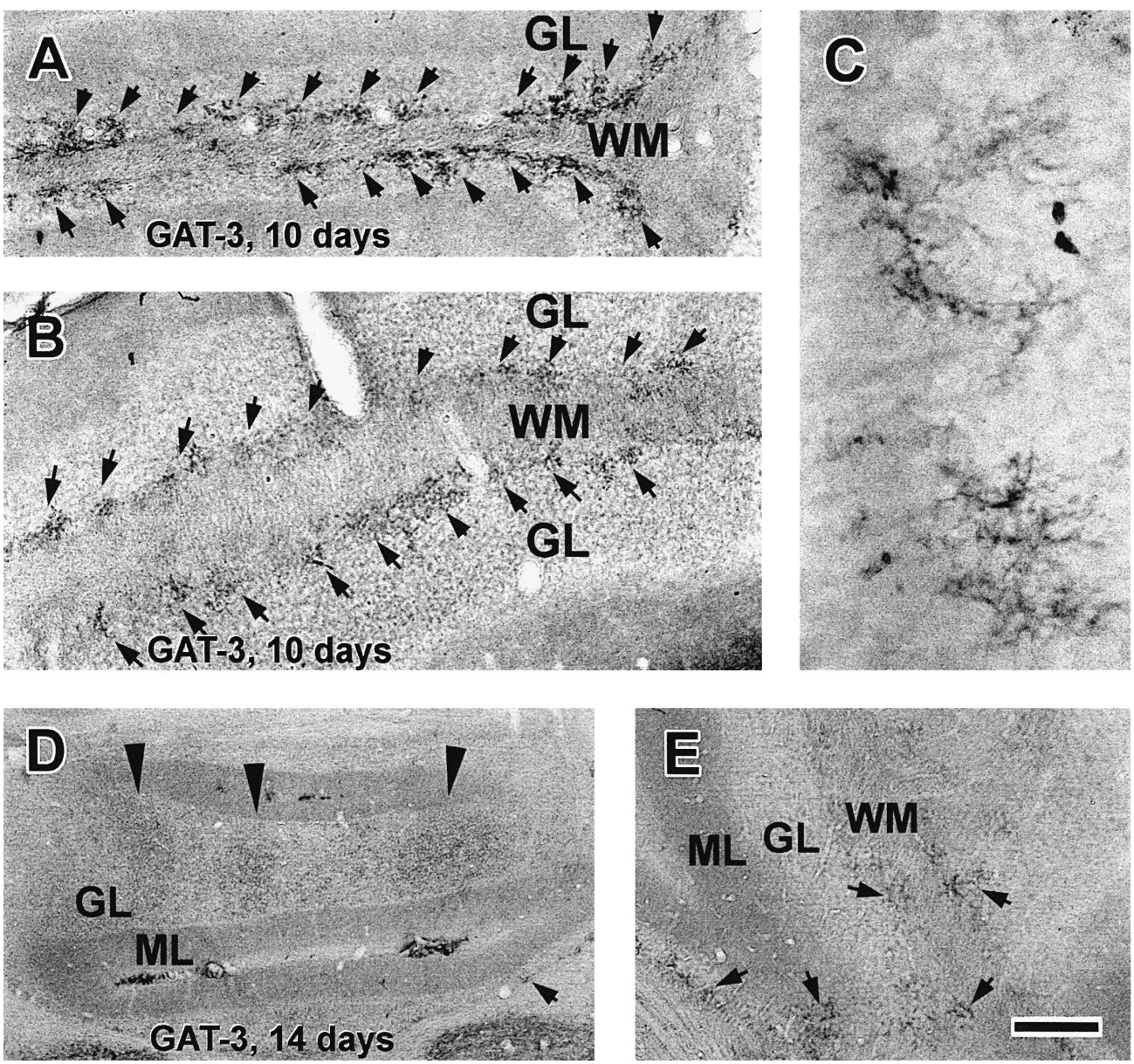

Fig. 3. GAT-3 immunoreactivity in the rat cerebellar cortex at P10 (A–C) and P14 (D, E). (A) and (B) show immunolabeled cellular profiles (small arrows) organized in regularly spaced clusters along the border of the cortex and white matter (WM) in the vermal and hemispheric lobules, respectively. Light diffuse immunolabeling also occurs in the GL. (C) Is an enlargement of the GAT-3 immunoreactive cellular profiles to show that they are astrocytes. By P14, the astrocytic clusters are no longer distinct in the central vermal cortex (D) but are still found in the hemispheric lobules (E). Scale bar = 200 μm for (A, B, D, E) and 20 μm for (C).

cells indicated that they were astrocytes. Further, the labeling of astrocytes showed a periodicity with about 100 μm intervals along the long axis of the cerebellar folium. The astrocytic clusters within the same folium and between neighboring folia were often congruent with the transverse planes of these folia in both the vermis and hemispheres (Fig. 3A,B).

3.3. Immunoreactivity at P14

The distribution and intensity of GAT-1 immunoreactivity were greater at P14 than at P10 (Fig. 4). In the vermis, GAT-1 immunolabeled somata were found in almost all lobules. In the hemispheres, immunoreactive somata were present in the medial 1/3–3/4 portions of the lobules and varied according to the rostrocaudal level (Fig. 4A). GAT-1 immunoreactive processes and neuropil were detected in the cortex throughout all cerebellar lobules. The centroperipheral gradient of GAT-1 immunoreactivity remained evident mainly in the hemispheres at this age (Fig. 4A). The patchy pattern of GAT-1 immunoreactivity was found in the peripheral folia or foliar portions where the Purkinje cell somata and dendrites began to be immunolabeled (Fig. 4B).

The labeled somata in the cortex could be distinctly divided into different types at this stage (Fig. 5). These cell types included the Golgi and Purkinje cells identified earlier. The latter were now arranged in a monocellular layer (Fig. 5A). The Purkinje cells in the central vermal and medial hemispheric cortex had well-immunostained dendrites in the molecular layer, as well as axons that extended across the granular layer into the white matter (Fig. 5A). Numerous small immunoreactive somata were found in the Purkinje cell and molecular layers. One group of them was located within and adjacent to the Purkinje cell layer and had a round soma (Fig. 5A). These cells appeared to be the basket cells according to their laminar localization [50]. In the middle and outer portions of the molecular layer, another group of small-sized somata was lightly immunolabeled for GAT-1. These cells often had an oval soma with its long axis running parallel to the pial surface of the cerebellar folium (Fig. 5E). They appeared to be the stellate cells on the basis of their laminar distribution pattern and morphology [50].

In the hemispheric lobules especially their lateral portions, the expression of GAT-1 first appeared in the somata of basket and stellate cells before that of Purkinje cells, since immunolabeled small cells were found in the molecular layer but the underlying Purkinje cell somata were not immunostained (Fig. 5A). Furthermore, in the most lateral portion of the hemispheres, GAT-1 immunoreactive plexuses, presumably derived from basket cells, were readily present within and adjacent to the Purkinje cell layer, whereas neither the basket nor the Purkinje cell somata of the same cortical regions displayed GAT-1 immunoreactivity (Fig. 4AFig. 5B). In the latter regions the GAT-1 immunoreactive processes formed a thin layer outlining the somata and axon initial segments of the unlabeled Purkinje cells (Fig. 5B). On the other hand, the basket cell processes in the vermis and the medial hemispheric cortex were well-developed deep to the labeled Purkinje cells (Fig. 5C,D), forming a thick cup- or nest-like structure, the so-called pinceau [52–54].

At P14, the GAT-3 immunoreactive somal profiles of astrocytes seen earlier in the vermal and hemispheric cortex–white matter border became less distinct (Fig. 3D,E). Meanwhile, light immunoreactivity appeared in the neuropil of the granular layer in both the vermal and hemispheric cortex. Furthermore, a patchy pattern of this GAT-3 neuropil immunolabeling was observed in the granular layer in some vermal lobules, especially in their median portions (Fig. 3D).

3.4. Immunoreactivity at P21

At P21, GAT-1 immunoreactive somata and processes (Fig. 6C–F) were present in all vermal and hemispheric regions. The most important developmental change at this age was the decrease in GAT-1 immunolabeling within the somata of all cerebellar GABAergic neurons. For the Purkinje cells, a small amount of immunoreactivity remained in the somal cytoplasm, whereas their dendrites in the molecular layer and axons in the granular layer and white matter were very faintly stained or no longer visible (Fig. 6B–D). Within the somata, the nuclear region had a relatively stronger immunolabeling than the cytoplasm, giving these cells a fried-egg appearance (Fig. 5C,D). The zonal pattern of GAT-1 immunoreactivity in the Purkinje cells was still recognizable at this stage but was much less distinct than at P10–P14 (Fig. 6F). Similar to the Purkinje neurons, the basket and stellate cells also contained a small amount of GAT-1 immunoreactivity in their somal cytoplasm (Fig. 5E). Immunolabeled Golgi-like neurons in the granular layer were not found at this age in any region of the cerebellum. In contrast to neuronal somata, the GAT-1 immunolabeling in the neuropil was increased in all layers of the cortex but it was reduced in the white matter. It should be noted that the most darkly labeled profiles in the cortex at this stage were the pinceaux (Fig. 5C,D,F).

GAT-3 immunoreactivity at P21 was localized exclusively to the neuropil in almost the entire vermal and hemispheric cortex (Fig. 7A,B). Only a few immunolabeled astrocytic somata were seen in the vermal folium adjacent to the fourth ventricle (arrowheads in Fig. 7C). The staining intensity in the granular layer was increased to moderate levels, whereas that in the molecular layer was considerably light at this age.

3.5. Immunoreactivity at P30–P60

At P30, a major developmental change in GAT-1 immunoreactivity was the complete loss of immunolabeling in the somata of the Purkinje, basket and stellate cells (Fig. 8A–D). Another change was a further increase in size and

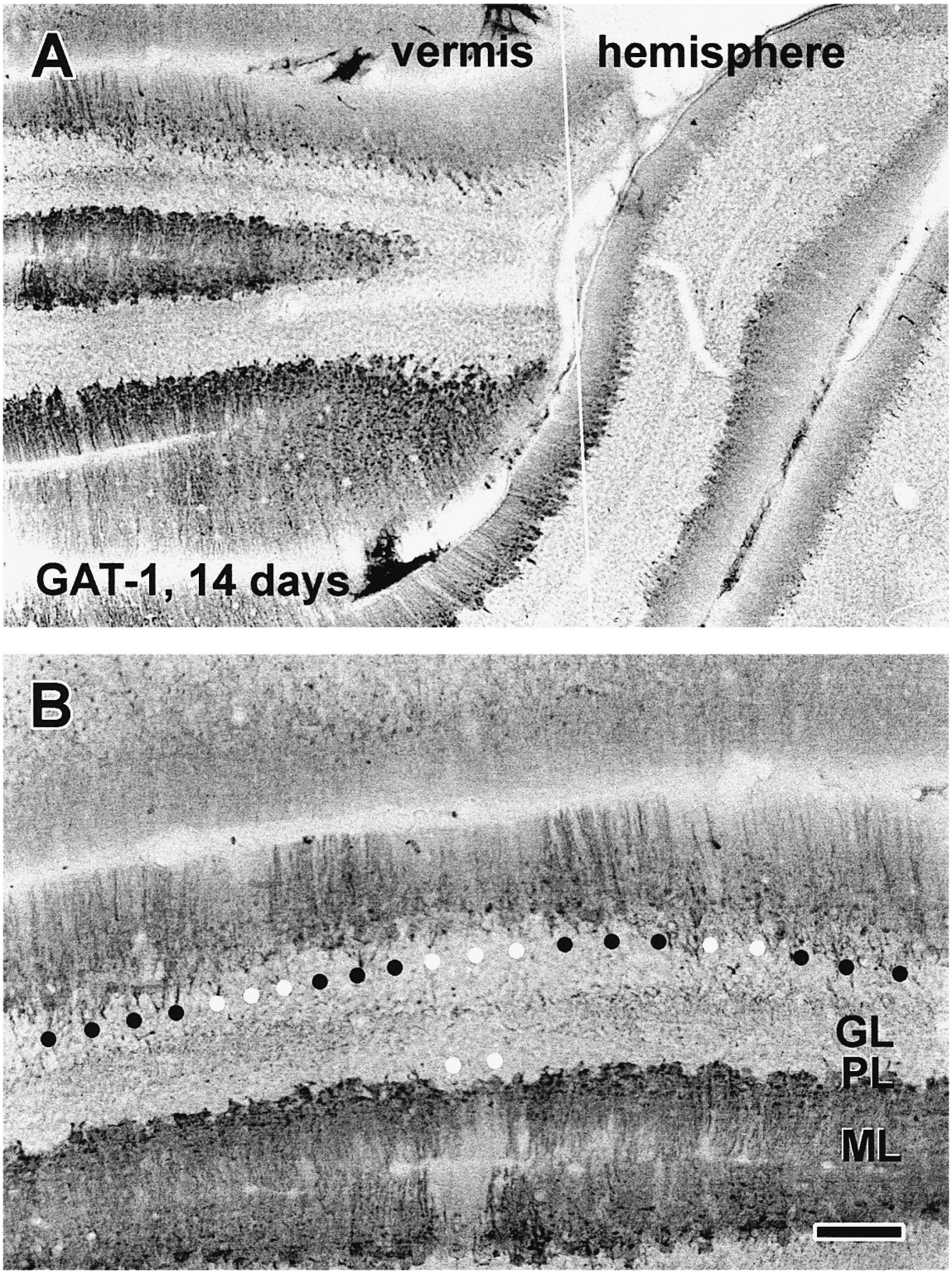

Fig. 4. Distribution of GAT-1 immunoreactivity in the rat cerebellar cortex at P14. (A) Shows that immunolabeled somata are found in most vermal lobules as well as the medial portions of the hemispheric lobules (white line indicates the border of the vermis and hemisphere). (B) Shows a banding pattern of the immunolabeled Purkinje cell somata and dendrites, which is found in the hemispheric cortex where the Purkinje cells are beginning to express GAT-1. Scale bar = 500 μm in (A) and 200 μm in (B).

immunostaining of the pinceaux that were now present in all vermal and hemispheric lobules. The overall staining intensity in the molecular layer was also increased, but the labeling did not appear in the dendrites of Purkinje cells (Fig. 8D), and was lighter than that in the pinceaux. By P45, an adult pattern of GAT-1 immunoreactivity was

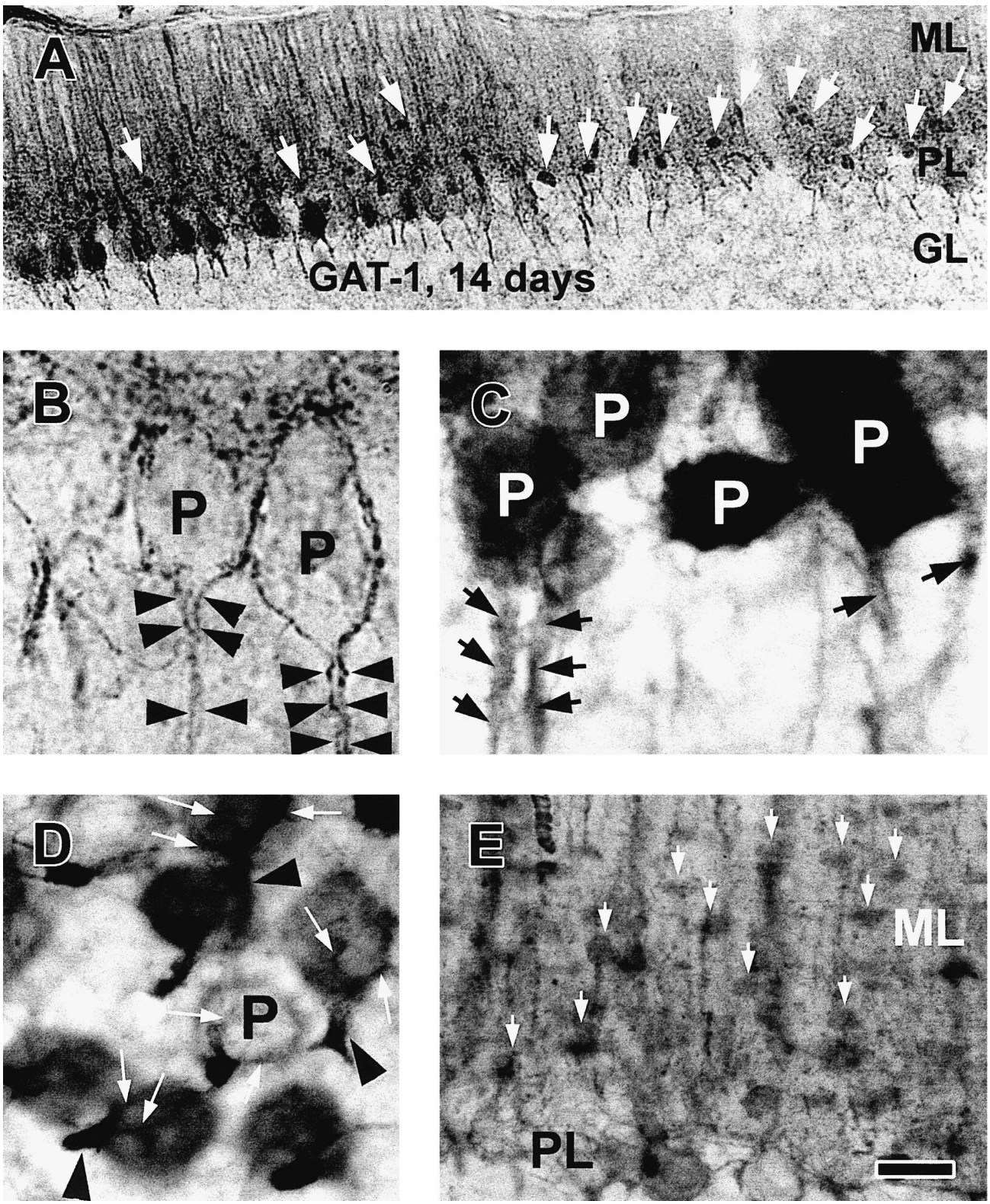

Fig. 5. Details of GAT-1 immunolabeled profiles at P14. (A) Shows that the expression of GAT-1 in the somata of basket (white arrows) and stellate cells slightly precedes that in the Purkinje cells in the hemispheric cortex. (B) Shows the initial appearance of GAT-1 immunoreactivity in the axon terminals (arrowheads) of basket neurons in the peripheral cortex where the Purkinje cell bodies (P) are not yet immunostained for GAT-1. (C) Shows that the Purkinje cells (P) in the medial hemispheric cortex express GAT-1 immunoreactivity within their somata and axons (arrows). (D) Shows well developed cup-like formations (pinceaux) located deep to and surrounding the somata of the Purkinje cells. These structures are thick (arrowheads) at the bottom of the Purkinje cells and have branches (white arrows) extending upwards. (E) Shows round and fusiform labeled cells in the middle and upper portions of the molecular layer representing stellate neurons (white arrows). Scale bar = 100 μm for (A), 50 μm for (E) and 20 μm for others.

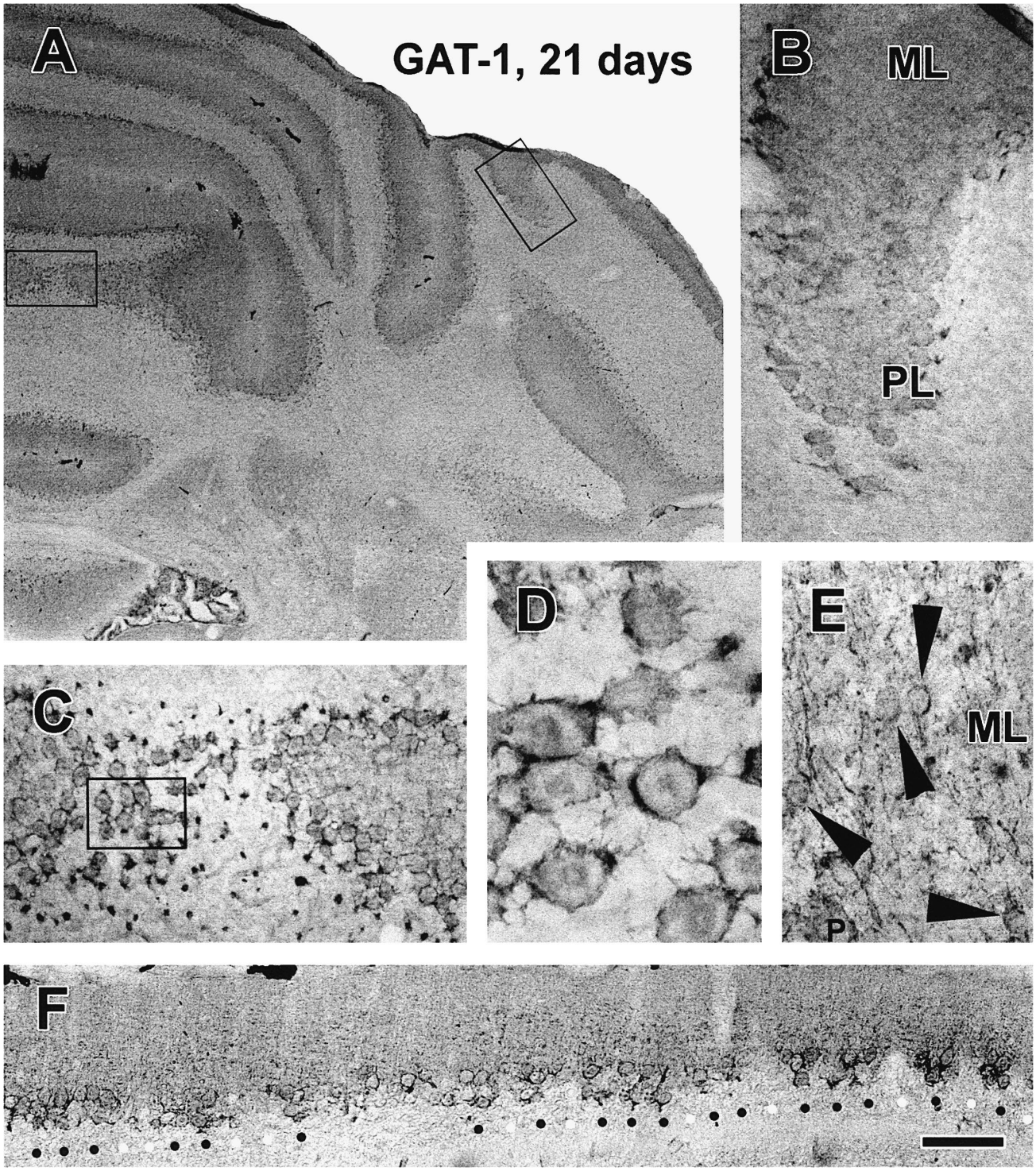

Fig. 6. GAT-1 immunoreactivity in the rat cerebellar cortex at P21. (A) Shows that at this age a homogeneous pattern of GAT-1 immunolabeling occurs throughout the cerebellar cortex. The boxed regions in (A) are enlarged in (B) for hemispheric and (C) for vermal cortices. In both areas, the Purkinje cell somata are immunostained for GAT-1 much lighter than at the earlier developmental stages (cf. Fig. 5). The boxed region in (C) is enlarged in (D) with a 90° rotation, to show that the nuclei of Purkinje cells display more immunoreactivity than the cytoplasm. (E) Shows that the GAT-1 immunoreactivity in the basket and stellate cells (arrowheads) are also decreased at this age. (F) Illustrates the banding pattern of alternating zones with labeled and unlabeled Purkinje cell somata is recognizable in some cortical regions (F). Scale bar = 500 μm for (A), 200 μm for (B, C, F) and 50 μm for (D, E).

completed in the rat cerebellum (Fig. 8E–F). The pinceaux appeared as thick cup-like structures inferior to the Purkinje cell somata which were GAT-1 immunonegative in all cerebellar regions (Fig. 8F). The labeling in the molecular layer became as dark as that in the pinceaux, and was packed with dense and evenly-distributed immunoreactive

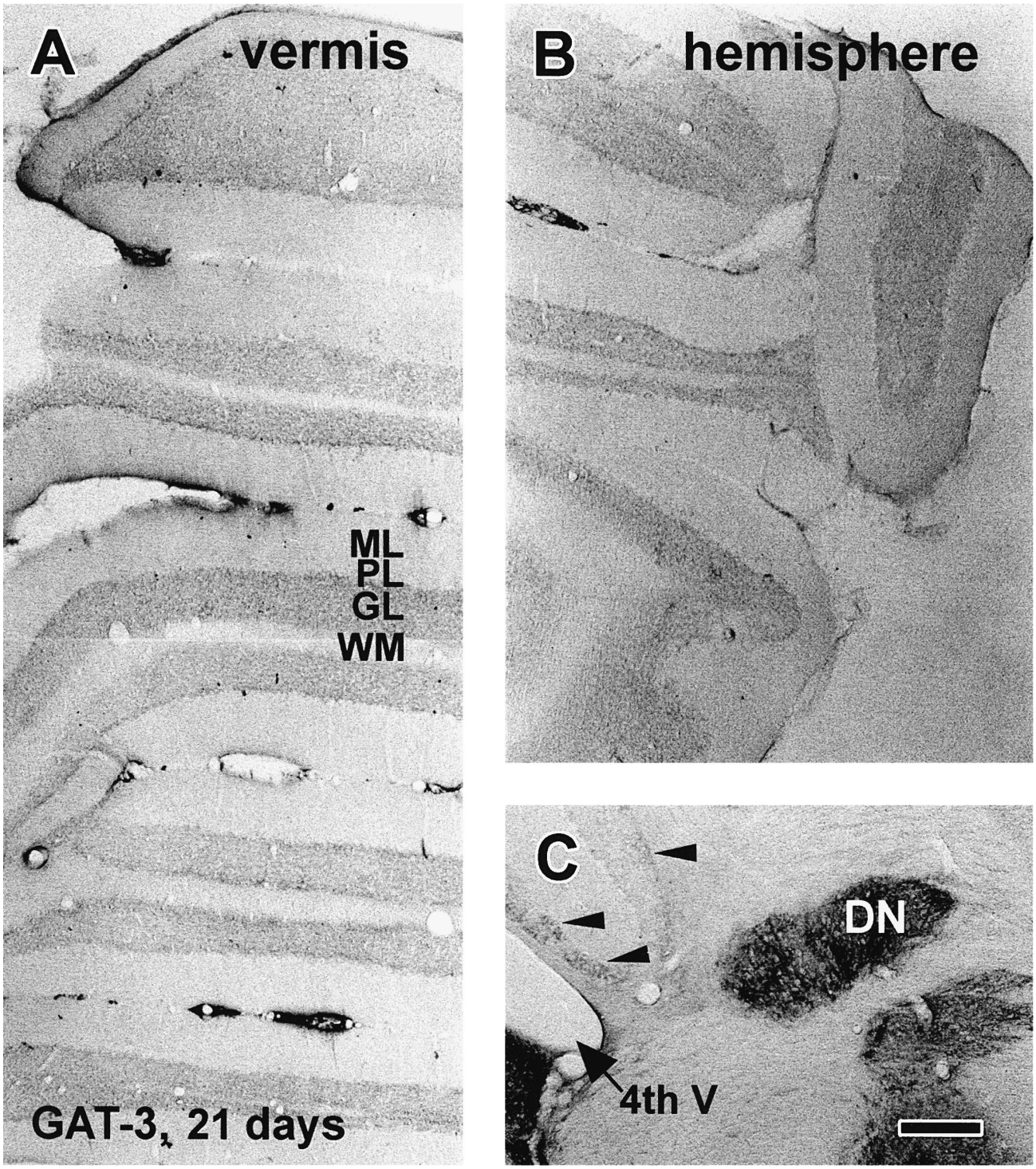

Fig. 7. GAT-3 immunoreactivity in the rat cerebellar cortex at P21. The distribution pattern of GAT-3 immunolabeling is similar for the vermal (A) and hemispheric (B) cortices, with diffuse neuropil labeling in mainly the GL. In the most ventral vermal folium at the roof of the fourth ventricle (4th V), a few stained astrocytic clusters (arrowheads) are present (C). The dentate nucleus (DN) is darkly stained for GAT-3. Scale bar = 500 μm.

neuropil. The granular layer consisted of lightly and evenly immunostained puncta that were probably associated with the cerebellar glomeruli (Fig. 8F).

An adult pattern of GAT-3 immunoreactivity in the cerebellar cortex was also completed through P30–P45 (not shown), which was largely similar to that at P21. This pattern was characterized by an exclusive neuropil immunolabeling throughout the cerebellar cortex. The staining intensity was heaviest in the granular layer, followed by the molecular layer, and finally the Purkinje cell layer. The distribution of GAT-3 immunoreactivity was homogeneous across each of the three cortical layers.

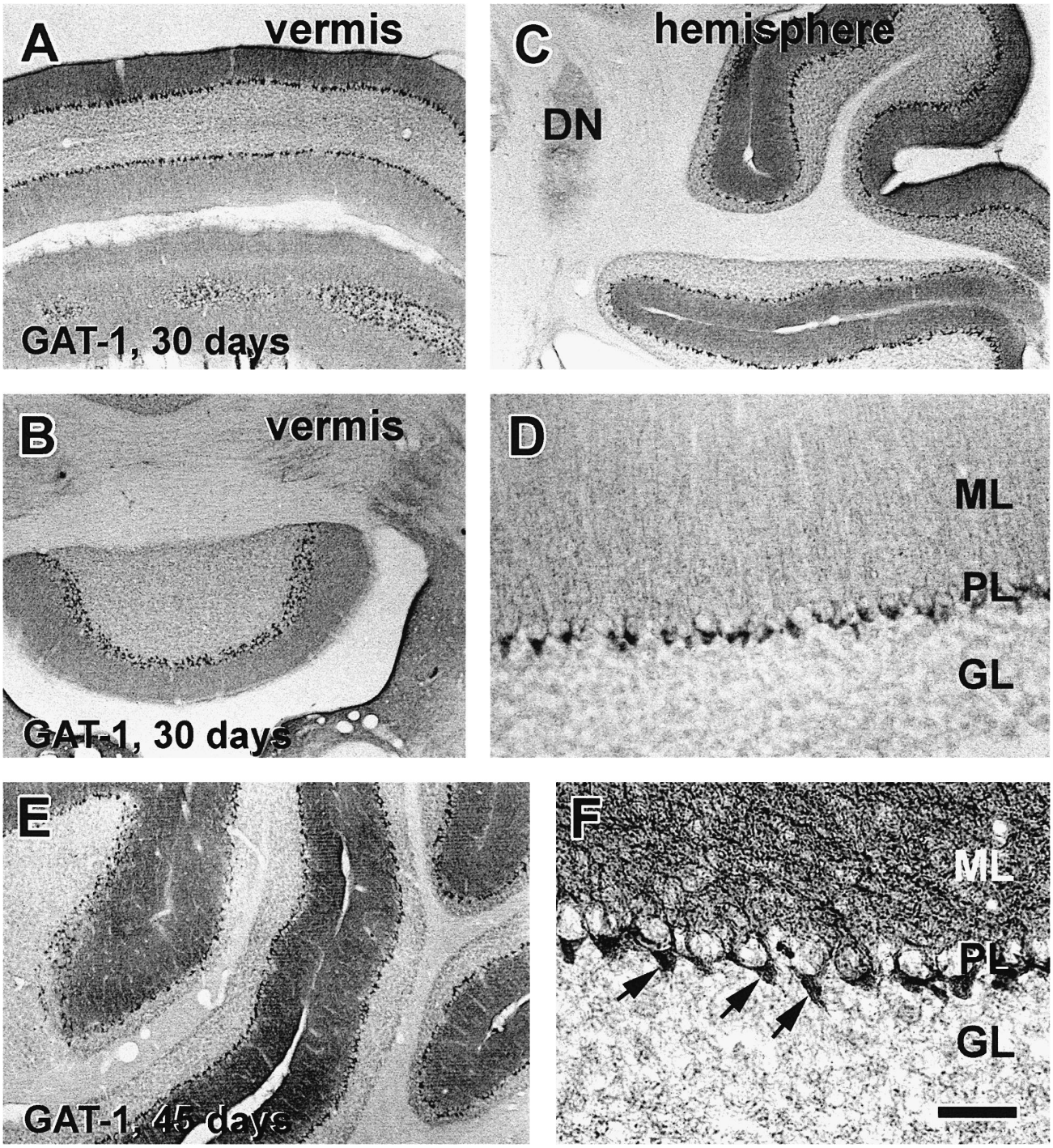

Fig. 8. GAT-1 immunoreactivity in the rat cerebellar cortex at P30 (A–D) and P45 (E, F) to show the establishment of a mature labeling pattern. (A, B, C) Are from the most dorsal vermis, most ventral vermis and the most lateral hemispheric region, respectively. Note that the immunoreactivity is mainly localized to the basket cell axonal plexuses and the molecular layer and that there is no banding pattern in these regions. At high magnification (D), the pinceaux deep to the immunonegative Purkinje cell somata are darkly stained, whereas the labeling in the molecular layer is moderate, and that in the granular layer is light. By P45 (E, F), the immunolabeling in the molecular layer is as dark as that in the pinceaux (arrows), while that in the granular layer remains light and appears in groups of small puncta. Scale bar = 500 μm for (A, B, C, E), 250 μm for (D) and 150 μm for (F).

3.6. Immunoreactivity in the deep cerebellar nuclei

The deep cerebellar nuclei showed immunoreactivity for both GAT-1 and GAT-3 at all ages examined in the present study. Between P7–P14, GAT-1 immunoreactive profiles in the dentate nucleus were present in the neuropil, including axonal processes and large puncta, but not cell bodies (Fig. 9A,B). The GAT-1 immunolabeled puncta were more darkly labeled than other labeled profiles. GAT-1 immunoreactive axonal processes were observed in

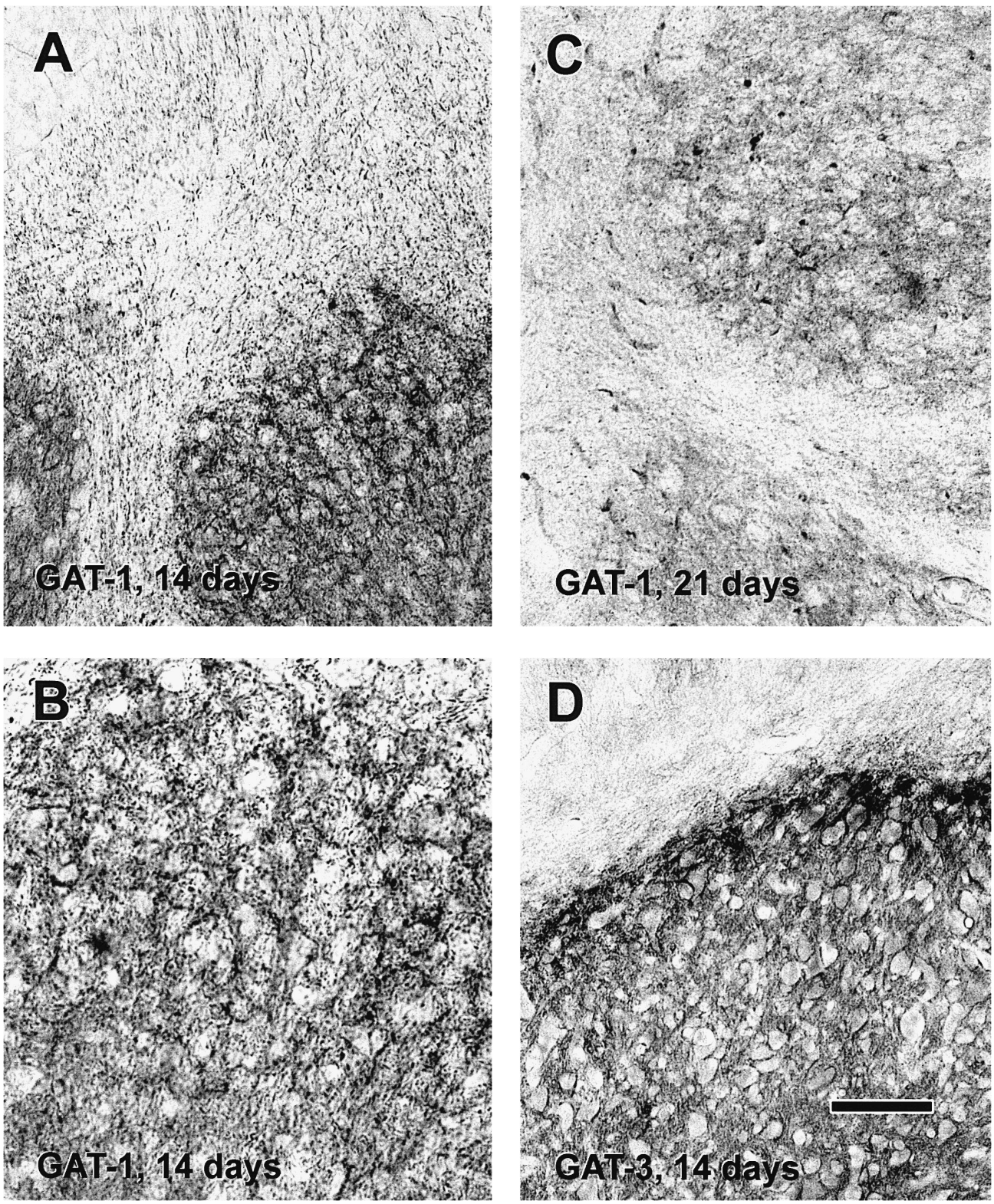

Fig. 9. Distribution of GAT-1 and GAT-3 immunoreactivity in the dentate nucleus of the rat cerebellum. (A) Shows GAT-1 immunoreactive axons at P14 that are organized in fiber bundles running in the white matter and invading the dentate nucleus (lower right corner). (B) Is an enlargement of this nucleus and shows numerous puncta that represent axon terminals which surround the GAT-1 immunonegative neuronal somata. (C) Shows that no GAT-1 immunoreactive axonal processes are present at P21 in the white matter, and no labeled puncta are within this deep nucleus, only a diffuse light neuropil staining occurs in this region. (D) Shows the pattern of GAT-3 immunolabeling in the dentate nucleus at P14. Note the heavily labeled neuropil. Scale bar = 200 μm for (A) and 100 μm for others.

the cerebellar white matter, invaded the deep nuclei, and were sometimes continuous with the puncta (Fig. 9A). At P21–P45, these axonal processes were not recognizable, and the axonal puncta within the deep nuclei also disappeared, resulting in a light and homogeneous immunolabeling in neuropil over this nucleus (Fig. 9C). GAT-3 immunoreaction product in this region was localized only to the neuropil at all ages (Fig. 7CFig. 9D), and displayed little change during the period examined (P0–P60).

4. Discussion

The present study indicates that the immunoreactivity for GAT-1 and GAT-3 appears in the rat cerebellar cortex around the end of the first postnatal week. The expression of these two GATs follows a similar spatiotemporal sequence in that the immunolabeled profiles appear first in the central vermal folia, then spread rostrally and caudally (as well as dorsally and ventrally) in the vermis and laterally to the hemispheres with increasing age. While the immunoreactivity for GAT-1 and GAT-3 is present throughout the cerebellum by P14, the overall staining intensity continues to increase until P45 at which time an adult distribution pattern is reached. During development, most immunoreactivity for GAT-1 and GAT-3 is found in the neuropil. However, the GABAergic neurons, including the Purkinje cells, transiently display GAT-1 immunoreactivity in their somata, dendrites and axons between P7 and P21, while the somata of astrocytes are distinctly immunostained for GAT-3 between P7 to P14.

4.1. Transient expression of GAT-1 in Golgi, basket and stellate cells

A major finding of the present study is that GAT-1 immunoreactivity is transiently expressed in the somata and dendrites of Golgi, basket and stellate neurons in the developing rat cerebellar cortex. In addition, GAT-1 immunoreactivity was found in the axonal processes during a restricted developmental period. Previous studies of GAT-1 in the adult cerebellum showed only labeling of axon terminals of these cells [54]. These three cerebellar GABAergic neurons lose GAT-1 immunoreactivity in their somata at P30, but maintain labeling of their axon terminals into adulthood. This finding for cerebellar GABAergic interneurons is consistent with the presence of strong GAT-1 immunolabeling of the basket plexus and pinceaux in light microscopic preparations as well as the immunolabeling of presynaptic terminals in the cerebellar cortex in electron microscopic preparations [52–54]. The developmental change in the localization of GAT-1 immunoreactivity in these cerebellar interneurons is also consistent with the finding of a transient expression of GAT-1 in the somata of GABAergic interneurons in the cerebral cortex [66].

4.2. Transient expression of GAT-1 in Purkinje cells

Our results show that Purkinje cells express GAT-1 in the entire neuron, including somata, dendrites, axons and axon terminals during a restricted time of development. Thus, labeled axons were in the white matter and entered the deep cerebellar nuclei where virtually all neurons were encased by GAT-1 immunoreactive presynaptic terminals. This latter observation contrasts with the adult labeling pattern of GAT-1 in the deep cerebellar nuclei where no immunolabeling for GAT-1 is found in Purkinje cell axon terminals [54]. Consistent with this adult pattern of GAT-1 labeling are results showing that mature Purkinje cells do not accumulate [^{3}H]GABA in vivo and in vitro [7,27,34]. Therefore, the Purkinje cells lose their GABA transport system from the second postnatal week, a time when the motor coordination function in rats develops rapidly.

The presence of GAT-1 in *immature* Purkinje cells indicates that these neurons transiently possess the ability for GABA uptake. Indeed, previous studies showed that Purkinje cell precursors from embryonic mouse cerebellum accumulate [^{3}H]GABA [23]. Furthermore, large, Purkinje cell-like neurons prepared from the rat cerebellum at P2 show [^{3}H]GABA uptake through the second to 15 days in culture [36]. Another study of the developing rat cerebellum used a tissue culture system that took cerebellar cortical explants from newborn pups and maintained them in vitro for 7 days [59]. It was shown in these cultures that large neurons with light and electron microscopic features of Purkinje cells were heavily labeled with [^{3}H]GABA. It is of interest that the Purkinje cells in the latter two rat studies are similar in age to those that express GAT-1 in the present study. However, to the best of our knowledge, there is no direct evidence demonstrating GABA uptake in developing Purkinje cells in vivo, because all previous [^{3}H]GABA accumulation studies in the developing brain did not provide data about the precise cellular localization of the labeling in cerebellar neurons [12,65].

It needs to be noted that a recent study in the developing mouse cerebellum did not detect GAT-1 mRNA signal in immature Purkinje cell bodies [19]. The discrepancy between the in situ hybridization data in the above investigation and the immunocytochemical findings in our present study might be because of a species difference in GAT-1 expression in developing Purkinje cells as rats were used in our study.

4.3. GAT-3 immunoreactivity in the somata of immature astrocytes

The present observation in the developing rat cerebellum indicates that GAT-3 immunoreactivity appears in somata of astrocytes between P7–P21. This finding is consistent with our earlier electron microscopic result that GAT-3 immunoreactivity is present in small amounts in the somata of adult astrocytes [54]. However, the current

study demonstrates that GAT-3 immunoreactivity is distinctly present in the somata as well as the processes of astrocytes in the immature cerebellar cortex at the light microscopic level. Thus, the expression of GAT-3 in astrocytes appears to be initially concentrated in the cell bodies and primary processes and then is evident in distal processes in the adult.

4.4. Transient banding pattern of GAT-1 and GAT-3 immunoreactivity

An interesting finding of this study is the banding pattern of GAT-1 and GAT-3 immunoreactivity in the rat cerebellar cortex during development. The present and previous studies indicated that such a pattern of immunolabeling did not exist in the adult cerebellar cortex [52–54]. For GAT-1, the zonal organization is derived from an uneven immunolabeling of Purkinje cell somata and dendrites in the tangential direction across the cerebellar folia. For GAT-3, it is related to a regularly spaced clustering of astrocytic somata. Thus, the banding patterns of GAT-1 and GAT-3 immunolabeling are not related to each other, though both exist transiently during development.

The mammalian cerebellum is compartmentalized functionally, anatomically, and biochemically as revealed by topographic mapping, tract-tracing and by using a range of molecular markers (for review see Refs. [24–26]). Numerous neuronal and some glial markers are expressed in the cerebellar cortex as parasagittal bands which in most cases are due to an uneven labeling of a probe to one or more cerebellar neuronal populations in one or more cortical layers [25,68–70]. In other cases, the banding is derived from a heterogeneous organization of the cerebellar fiber systems or certain types of cerebellar cells [24]. The latter includes astrocytes [49] and a recently identified cerebellar interneuron, the unipolar brush cell [67]. It is important to note that the GABAergic markers, GAD and GABA-B receptor, are expressed in the rat cerebellar cortex in a banding pattern during development and in adulthood [1,9]. The present study indicates that two additional GABAergic markers are transiently expressed in the cerebellar cortex in a zonal way during development.

4.5. Comparison of GAT expression with other GABA parameters

The cerebellar GABAergic neurons synthesize GABA at an early developmental stage, beginning in the embryonic period in the rat [12,19,21,42]. For example, the activity of the GABA synthesizing enzyme, glutamic acid decarboxylase (GAD), is detectable at embryonic day 15 and progressively increases until the end of the second postnatal month to achieve the adult level [12]. The Purkinje cells exhibit differential mRNA signal for the two GAD isoforms, GAD-65 and GAD-67, by P7 and P10, respectively [19]. Other cerebellar GABAergic neurons, including the Golgi, basket and stellate cells, express GAD-65 and GAD-67 mRNAs later than the Purkinje cells, often around P10 to P14. A remarkable increase of mRNA expression occurs immediately after P14 and continues to the end of the first postnatal month to reach a level that is slightly above the adult value [19]. The content of endogenous GABA exhibits a similar progressive pattern during this same developmental period, but its levels are non-proportionally higher than that of GAD activity [12,21,63]. As for the GABA transport system, [^{3}H]GABA uptake in whole cerebellar extracts is less than 50% of the adult value at birth and plateaus around 200% over adult levels between P8 and P21, and decreases toward adult levels at P28 [12]. The amount of GAT-1 mRNA in whole cerebellar preparations as determined by nuclease protection assay approximates the adult level for the first 10 postnatal days, increases up to 3-fold by P21 and maintains this level until P30, then is reduced to the adult level by P40 [65]. Taken together, although there is no simple relationship between the various GABAergic parameters, two developmental phenomena appear to be evident. First, there is a rapid increase of these markers beginning in the second postnatal week and continuing for about one month. Second, all parameters tend to be over-expressed during a period before the adult levels are achieved.

The data from the present study indicate that the developmental expression of GAT-1 and GAT-3 coincides well with that of these GABAergic parameters. In particular, both GAT-1 mRNA and [^{3}H]GABA uptake are extraordinarily high between the second and fourth postnatal weeks compared to adult values [12,65]. One can speculate that there must be an overactive GABA uptake system in the cerebellar cortex at this developmental stage. Indeed, our study indicates that GAT-1 immunoreactivity is transiently expressed in the somata of most GABAergic neurons of the cerebellar cortex during this same time span. Thus, the presence of GAT-1 in the cell body and dendrites of the immature GABAergic neurons may be, at least, part of the basis of an exceptionally strong GABA uptake activity in this brain structure during development.

4.6. Relationship between GAT expression and other developmental aspects

The centroperipheral sequence of the developmental expression of GAT-1 and GAT-3 in the rat cerebellar cortex is consistent with the general spatiotemporal order of histogenesis and neuronal maturation in the cerebellar cortex [40]. For example, Golgi and Purkinje cells are differentiated earlier than basket and stellate cells, and we found that at P7, the former two cells in the central vermis are immunolabeled for GAT-1, but the latter two are not yet labeled. However, it should be emphasized that the expression of GAT-1 in cerebellar GABAergic neurons occurs mainly after they complete their migration; so the sequence of GAT-1 expression among the cerebellar

GABAergic neurons does not always follow the order of neurogenesis. In fact, the basket and stellate cells in the hemispheric lobules express GAT-1 slightly in advance of the Purkinje cells of the same regions.

On the other hand, the expression of GAT-1 in cerebellar GABAergic neurons is well correlated with the differentiation of the dendritic and axonal processes of these neurons. A good example for this notion is the development of the pinceaux deep to the Purkinje cell bodies. At first, GAT-1 immunoreactive basket axon terminals form a thin layer around Purkinje cell somata and axon initial segments. Later, many of these terminals aggregate at the base of Purkinje cells to become a thick typical cup-like structure. Further, the axonal profiles of the Purkinje cells in the white matter and deep nuclei are distinctly immunolabeled for GAT-1 during the second to third postnatal weeks. Thus, it is obvious that GAT-1 is present in the developing axon terminals of cerebellar GABAergic neurons. The presence of GAT-1 at the terminal tips of the developing axons suggests that GAT-1 may be involved in GABA transport at the developing synaptic sites.

Finally, although the major role of GATs is for GABA uptake from the synaptic cleft and extracellular space, a body of evidence indicates that they may also be involved in synaptic and non-synaptic release of GABA in certain situations including during development [2–4,35,37]. Also, GABA release is observed in vitro from developing and even adult mouse cerebellar slices [35]. In general, GABA is believed to play an important neurotrophic role in neuronal development of the brain, which is mediated via excitatory, instead of inhibitory, ionic mechanisms, including calcium channels [10,46,51,60]. In particular, GABA seems to affect the morphological maturation as well as the expression of neuron-associated proteins of developing cerebellar neurons, especially the granule cells [41,46]. Thus, the possibility exists that the transient expression of GAT-1 in the cell bodies of GABAergic neurons is to release GABA from these neurons which may ultimately play a role in promoting differentiation and maturation of developing cerebellar neurons [66].

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