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DEVELOPMENTAL BRAIN RESEARCH

Research report

Immunoreactivity for GABA plasma membrane transporter, GAT-1, in the developing rat cerebral cortex: transient presence in the somata of neocortical and hippocampal neurons

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Abstract

The immunoreactivity for a γ-aminobutyric acid (GABA) membrane transporter, GAT-1, was examined in the neocortex and hippocampal formation of developing rats from the day of birth (postnatal day 0, P0) to the adult stage. The immunolabeling was mainly localized to the neuropil, but was also in a select population of cell bodies during a limited time period. Layers I and VIb of neocortex exhibited relatively high reactivity at birth, but diminished their staining with development. In contrast, GAT-1 immunoreactivity in the neuropil in the cortical plate and its derivatives was light at birth, but increased rapidly during the first 2–3 postnatal weeks in an inside-out order. An adult pattern with immunoreactive puncta more densely distributed in layers II to IV than the deeper layers was completed by P30–45. The neuropil reactivity in the hippocampal formation at P0 was greater than that in the neocortex, densely localized in a supragranular band, and less densely in the hilus of the dentate gyrus and the strata radiatum and oriens of the hippocampus. This pattern was basically maintained at later stages except that the immunoreactivity in the supragranular band diminished, whereas that in the subgranular zone was enhanced. A population of cell bodies morphologically characteristic of cortical and hippocampal interneurons was substantially immunolabeled for GAT-1 by P5 and remained until P30. At the electron microscopic level, GAT-1 immunoreactivity was localized mainly to axon terminals and astrocytes between P5 and P45, but was also found in neuronal somata and their dendrites between P5 and P30. Our data show a differential postnatal development of GAT-1 immunoreactivity in the rat cerebral cortex, including a transient presence of immunoreactivity in the somata of a subpopulation of cerebral interneurons and a developmental downregulation of GAT-1 expression in the earliest generated cortical elements (layers I and VIb). The findings in the present study suggest that GAT-1 expression in the neocortex and hippocampus may relate to the functional maturation of the GABAergic system.

Keywords: γ-Aminobutyric acid; GABA transporter; Interneuron; Astrocyte; Ontogeny; Cerebral cortex; Hippocampus

1. Introduction

The γ-aminobutyric acid (GABA) uptake mechanism plays an important role in the regulation of the magnitude and duration of GABA's synaptic action [9,19,21,26]. The GABA uptake system may also transport GABA into the extracellular space in a Ca^{2+}-independent, nonvesicular manner [1,3,28,31]. The GABA transporters have been cloned and classified into 4 members, GAT-1, GAT-2, GAT-3 and GAT-4, by differential amino acid sequences and pharmacological properties [9,15,21,25,32,58,70]. GAT-1 displays pharmacological features typical of a 'neuronal transporter': its GABA uptake is strongly inhibited by *cis*-3-aminocyclohexane carboxylic acid (ACHC) but not by β-alanine. GAT-2 and GAT-3 exhibit pharmacological properties often associated with 'glial transporters' whose GABA uptake is inhibited by β-alanine but not ACHC. GABA uptake by GAT-4 is inhibited by neither agent [9,15,19,26,32]. Antibodies directed against these proteins have been applied to study their tissue distribution, and GAT-1 and GAT-3 have been shown to be brain-specific [25,38,42,43,58].

GABA plays an important role in regulating the activity of principal neurons in the cerebral cortex [27,29,48]. GABAergic neurons have been well characterized in the neocortex and the hippocampal formation using immunocytochemistry to visualize GABA, and its synthetic enzyme, glutamic acid decarboxylase (GAD) [22,23,44,51,55,67]. Recent studies indicate that the major

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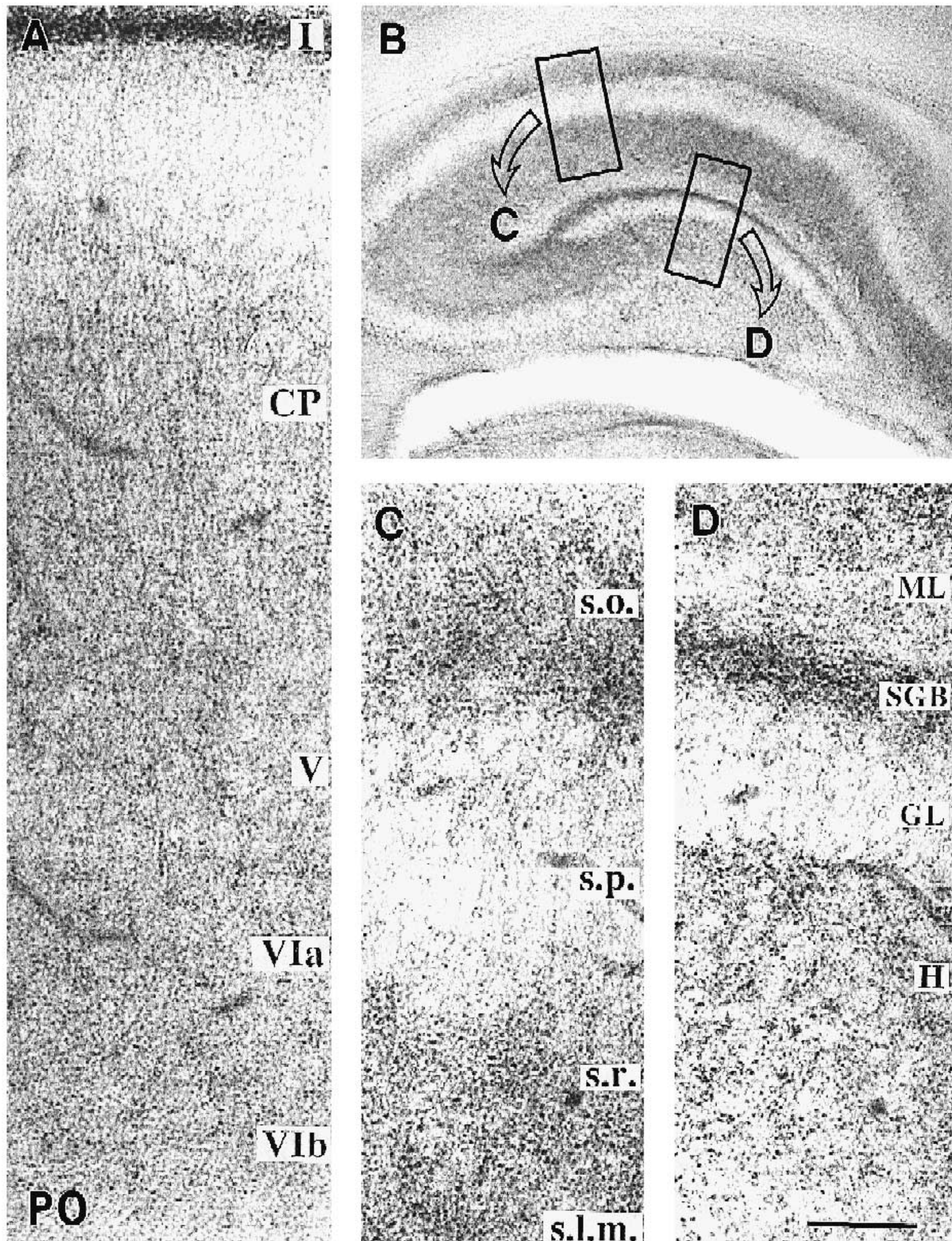


Fig. 1. GAT-1 immunoreactivity in the neocortex (A) and hippocampal formation (B–D) of a newborn rat (P0). (A): coronal section of parietal cortex shows strong GAT-1 immunolabeling in layer I, light reactivity in layer VIb and faint staining in other layers. B: low magnification coronal section of GAT-1 immunoreactivity in the hippocampus and dentate gyrus. (C) and (D) are enlargements of the two boxed regions indicated in (B), showing the details of the immunolabeling in CA1 area (C) and the dentate gyrus (D), respectively. The neuropil reactivity in the hippocampal formation is greater than that in the neocortex. Note the strongest immunoreactivity for the hippocampal formation within the supragranular band (SGB) in (B,D). Both the stratum pyramidale and granule cell layer demonstrate the faintest reactivity. CP: cortical plate; s.o.: stratum oriens; s.p.: stratum pyramidale; s.r.: stratum radiatum; s.l.m.: stratum lacunosum-moleculare; GL: granule cell layer; H: hilus. Scale bar = 125 μm for (A,B), and 50 μm for (C,D).

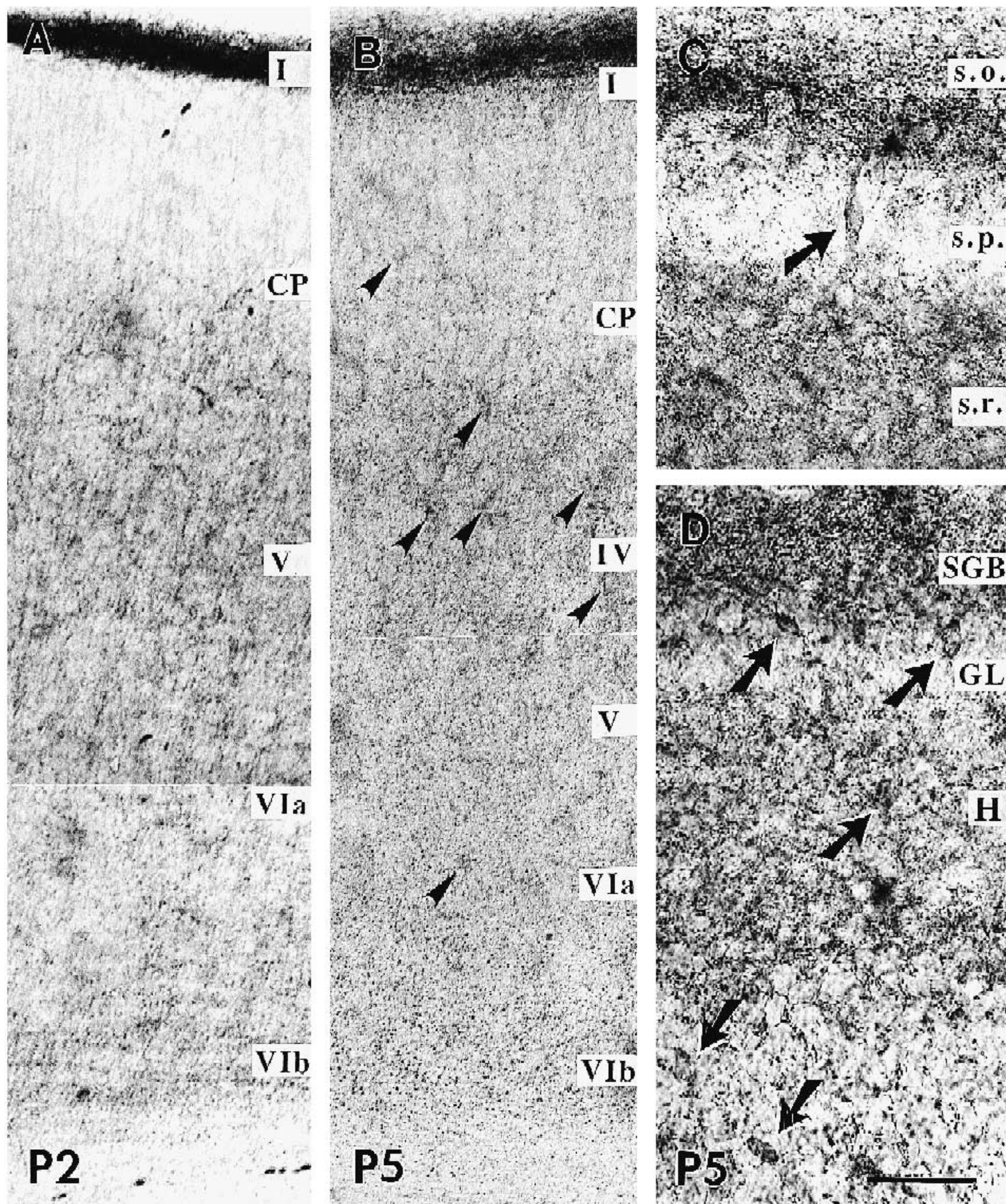


Fig. 2. Distribution of GAT-1 immunoreactivity in the cerebral cortex of the rat at P2 (A) and P5 (B–D). (A) shows that the neuropil immunoreactivity is increased in layer VIa to the deep part of the cortical plate as compared to P0, whereas layer I remains darkly stained. The prospective layers II–III are lightly labeled. (B) shows that punctate immunoreactivity is present throughout the neocortex at P5, and the staining in layer IV is denser than its neighboring layers. The staining in layers I and VIb is reduced to a certain degree as compared to the earlier ages. (C) and (D) illustrate the immunolabeling in the CA1 subfield and dentate gyrus at P5, respectively. The neuropil immunoreactivity in the hippocampal formation at this age is higher than that at P0. The punctate profiles are outside the cell bodies of the pyramidal and granule cell layers . Note the presence of GAT-1-immunoreactive somata in both the neocortex and hippocampal formation (arrows and arrowheads in B–D). Abbreviations as in Fig. 1. Scale bar = 125 μm for (A) and (B), 50 μm for (C) and (D).

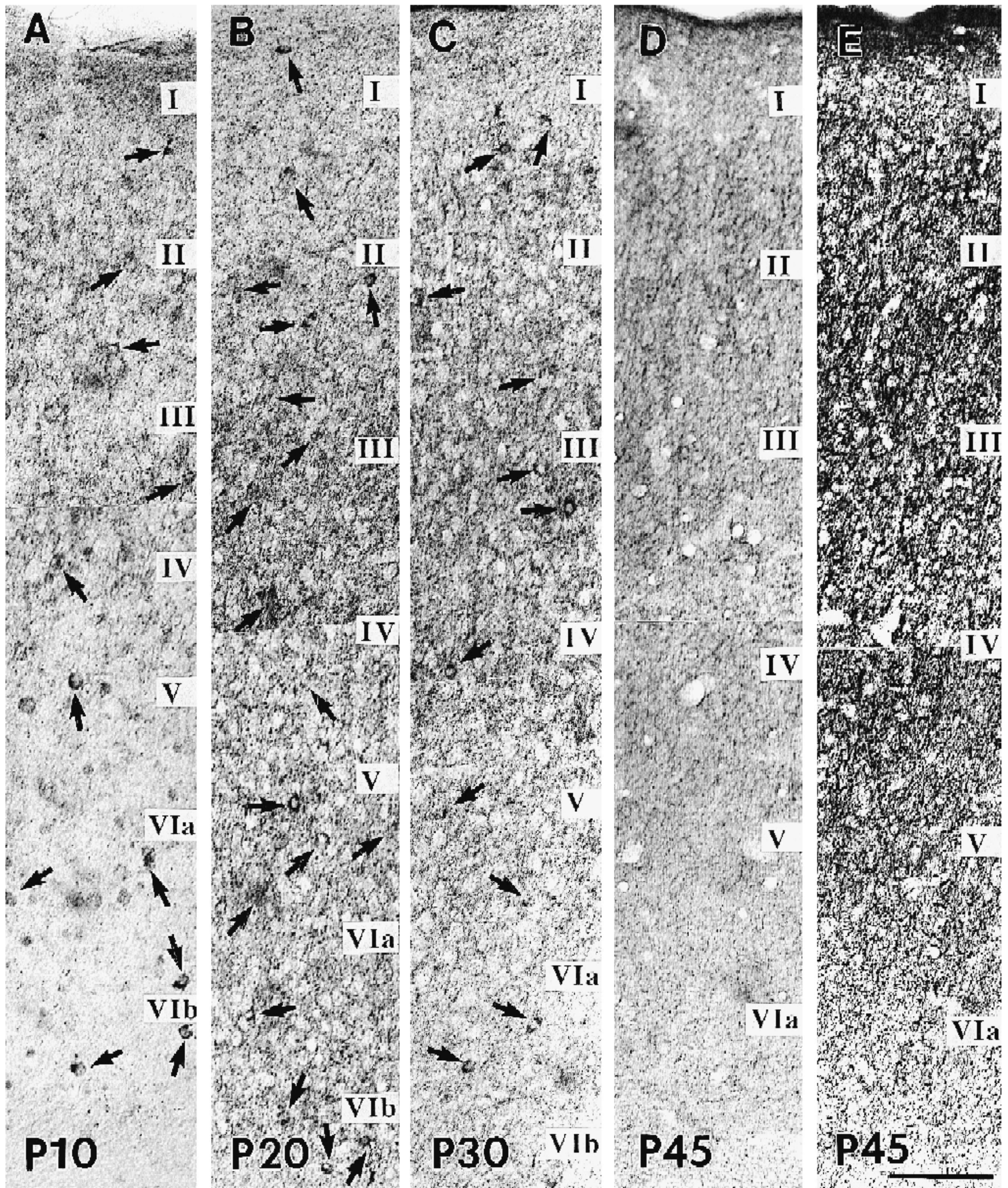


Fig. 3. GAT-1 immunoreactivity in the developing rat neocortex from P10 to P45 (A–E). Ages are marked for each microphotograph at the lower-left corner. Immunoreactivity is increased in layers II–VIa, notably during P10–P30 as compared to earlier ages. The neuropil labeling is present throughout the cortex, but is denser in layers II–IV. By P30–45, the laminar pattern and intensity of the punctate immunolabeling are stabilized. A number of distinctly labeled somata are present in all layers and white matter during P10–P30 as indicated by arrows in (A–C). The section shown in (E) was treated with a relatively high concentration (1:750) of the primary antibody (for other sections, 1:2000) and this resulted in an elevated neuropil reactivity but no somatic labeling. Scale bar = 125 μm.

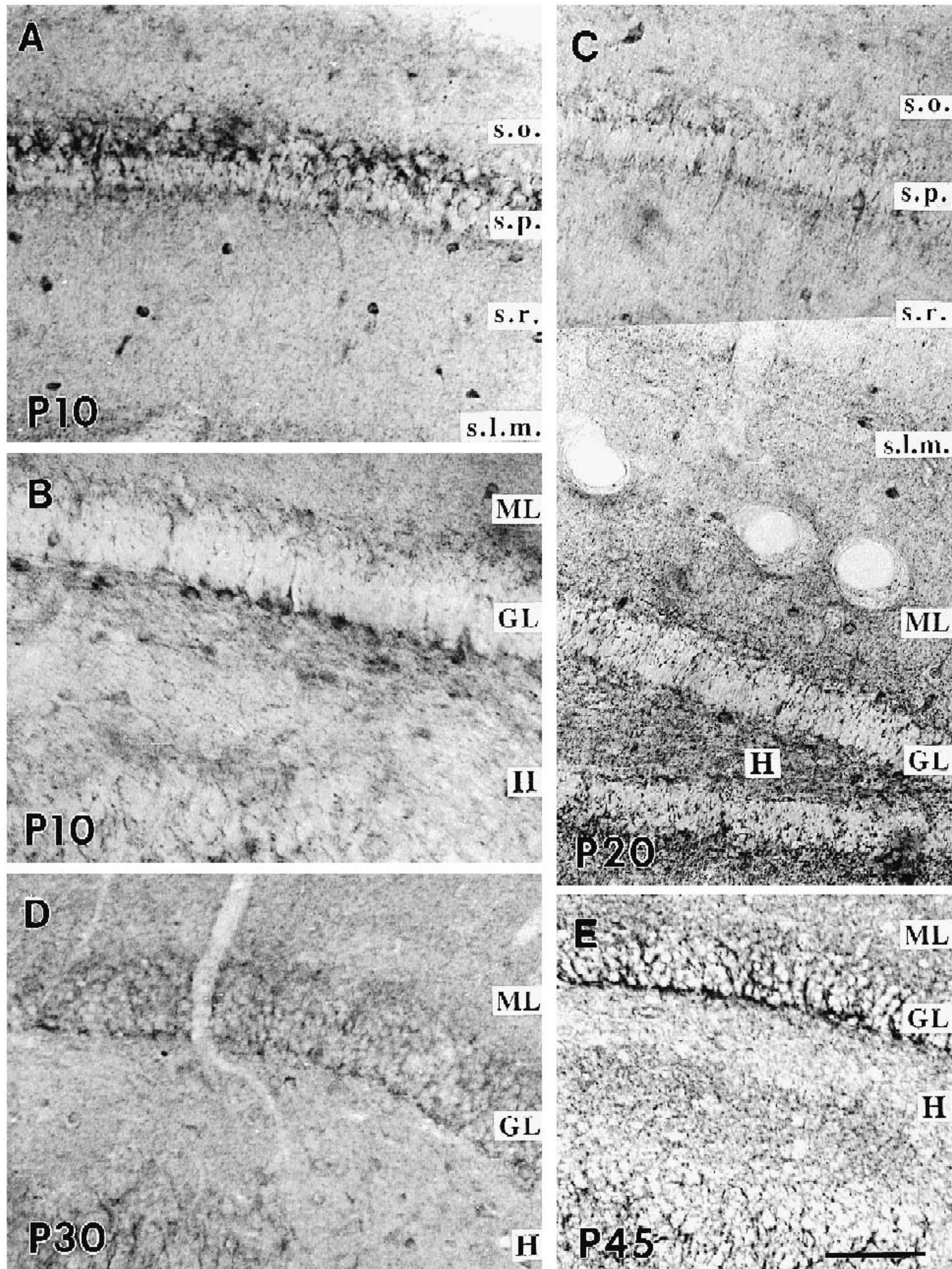


Fig. 4. GAT-1 immunoreactivity in the hippocampal formation of rats aged from P10 to P45 (A–E). Ages are marked for each microphotograph at the lower-left corner. The strong immunoreactive neuropil in the supragranular band seen at P0–P5 is no longer detected at P10 or older ages (cf., B–E with Figs. 1 and 2), whereas the immunoreactive puncta within, and at the lower borders of the pyramidal and granule cell layers increase during P10–P45. By P45, the strongest neuropil labeling is localized deep to the granule cell layer. Immunolabeled somata are seen in all strata of the hippocampus and dentate gyrus from P10–30, but not at P45. Abbreviations as in Fig. 1. Scale bar = 125 μm.

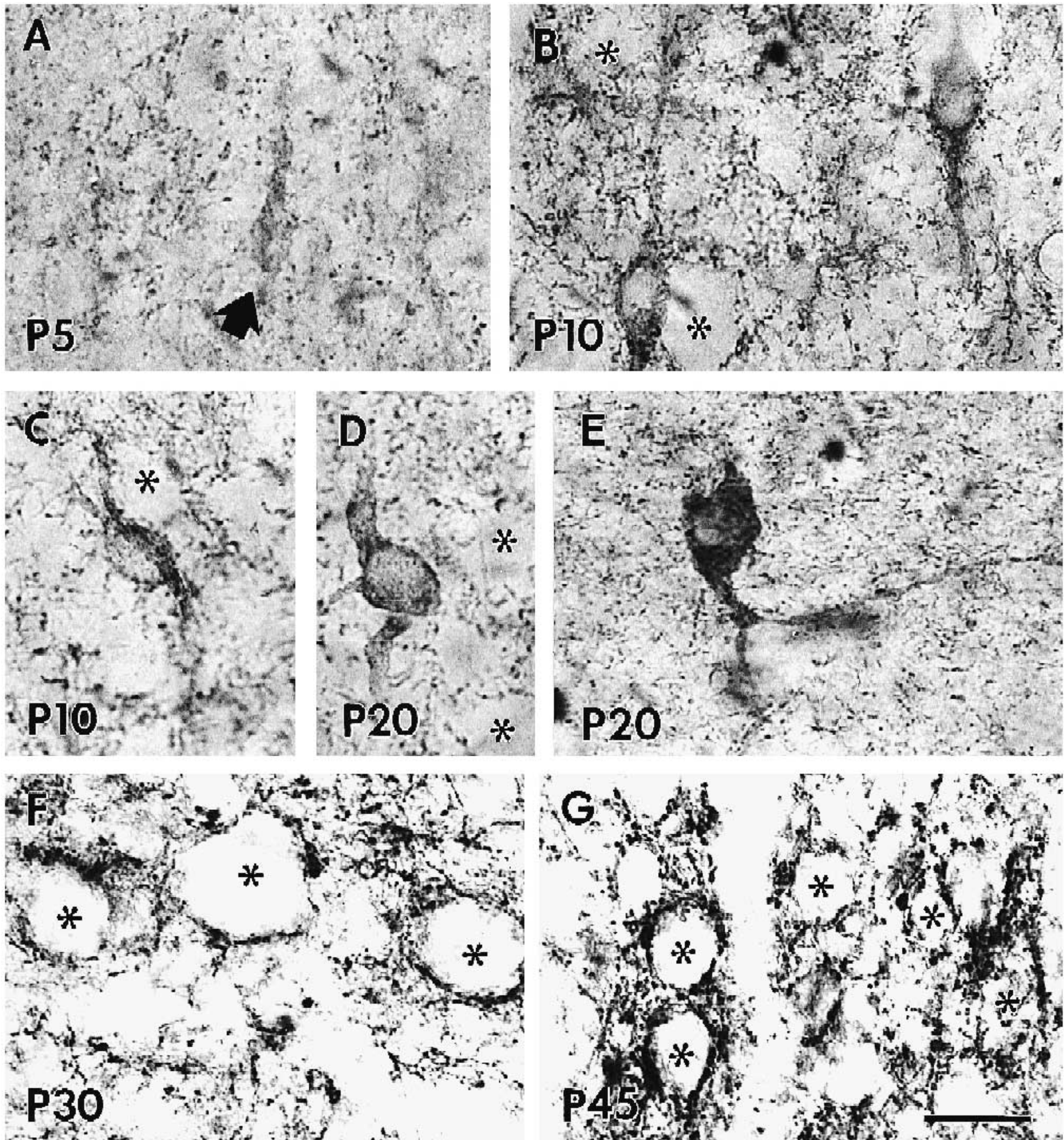
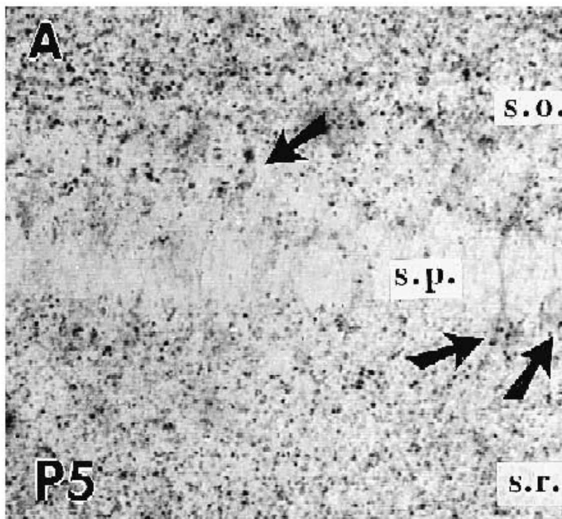
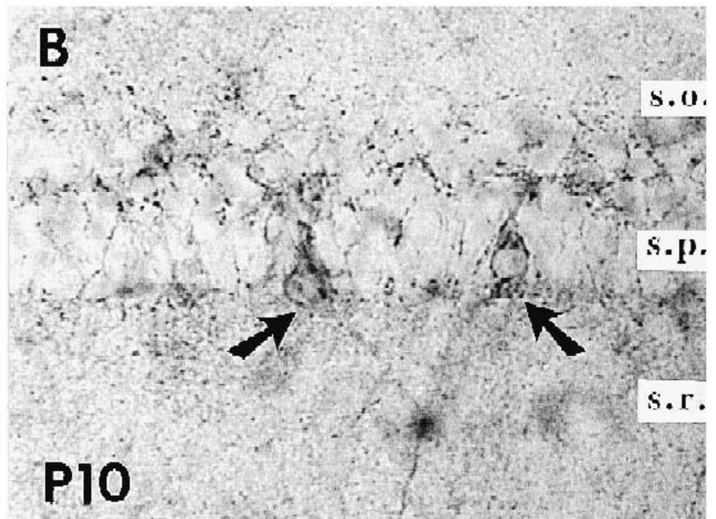


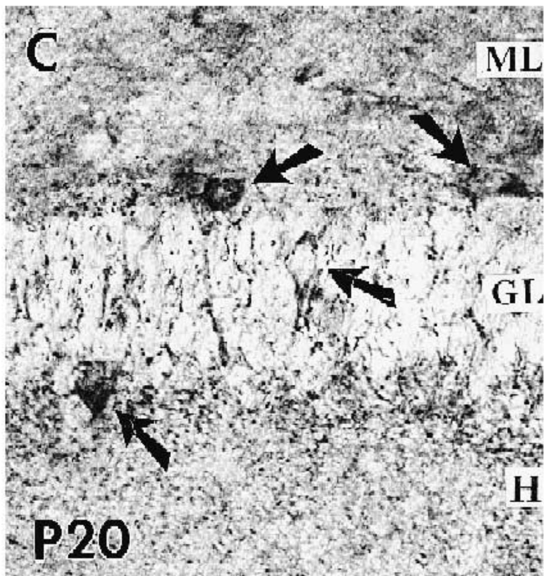
Fig. 5. Details of the morphology of GAT-1-immunoreactive puncta and somata in the developing rat neocortex. Ages are given for each microphotograph. Immunopositive somata (arrow) are identifiable at P5 (A), and become very distinct at P10–20 (B–E). The immunoreactive perikarya have varied shapes: three cells in (A,B) are bipolar, the cell in (C) may be bitufted and those in (D,E) are multipolar. F,G: the GAT-1-immunoreactive puncta apposed to the somata and dendrites of unlabeled pyramidal neurons (asterisks) are increased in number with age. All microphotographs were from layer IV. Scale bar = 25 μm.

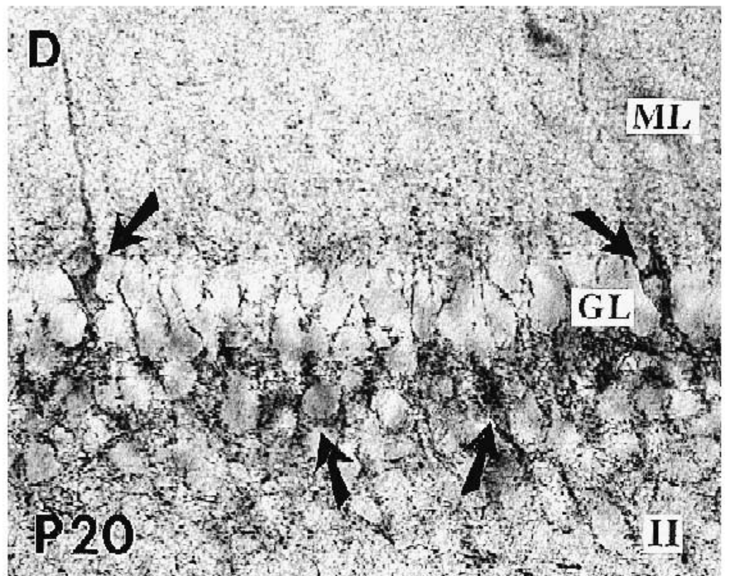
Fig. 6. Details of GAT-1-immunoreactive puncta and somata in the developing rat hippocampal formation. The immunoreactive puncta are diffusely distributed at P5 (A), and become more closely associated with the immunonegative somata and their dendrites in the cellular layers with increasing age (B–F). A number of positive somata within or near the pyramidal and granule cell layers (arrows) are apparently basket cells. Two types of basket neurons are displayed in (F): solid arrow indicates an inverted pyramidal basket neuron; open arrows indicate two basket cells at the hilar border of the granule cell layer. Scale bar = 50 μm for (A–E), 25 μm for (F).

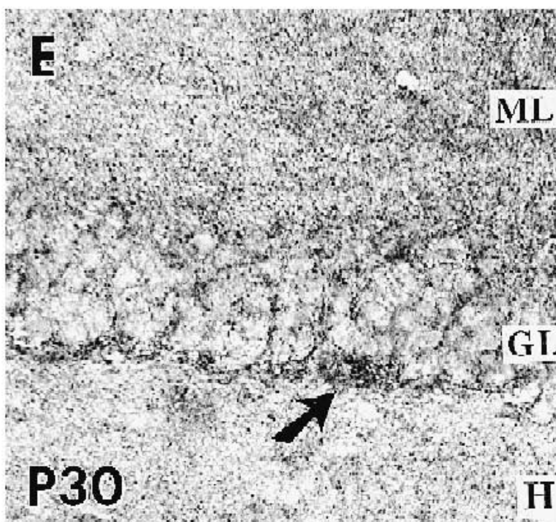
GABA transporter in the adult cerebral cortex is GAT-1 which is localized mainly to axon terminals and astroglia [38,42,43,58]. We have recently shown that immunolabeling for both GAT-1 and GAT-3 in the adult rat hippocampal formation is detected in glial cells, but only GAT-1 immunoreactivity is found in neurons[47]. Although it was

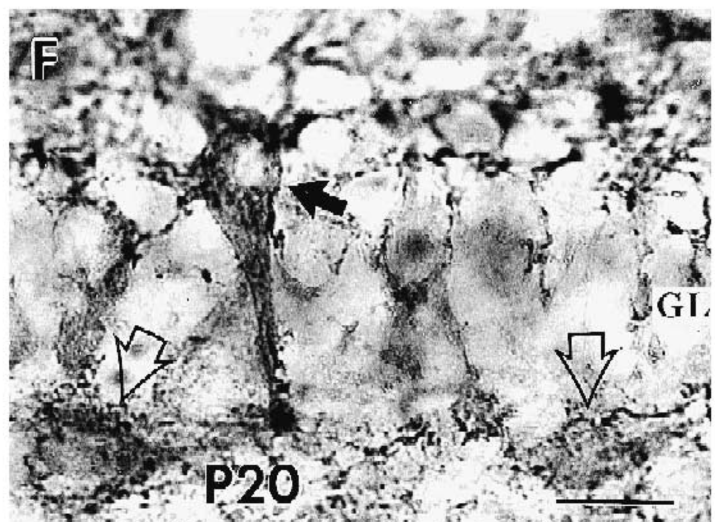












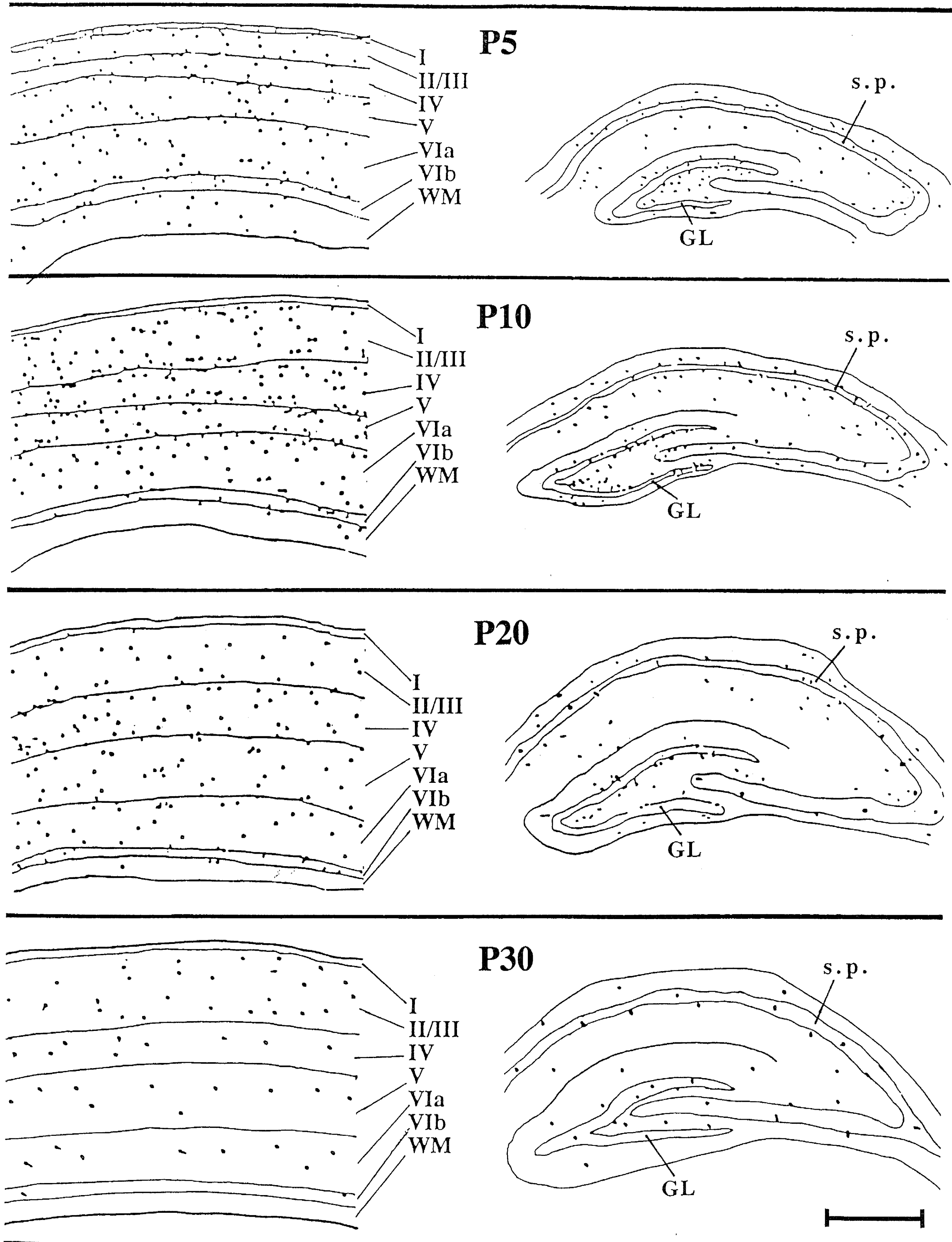


Fig. 7. Laminar distribution and relative numerical density of GAT-1-immunoreactive cell bodies in the neocortex and hippocampal formation of the rat from P5 to P30. There was virtually no somatic labeling detectable in the cerebral cortex of rats younger than P5 and older than P30. Data were obtained from the parietal cortex and underlying hippocampal formation. Each dot represents one immunoreactive soma obtained from a single representative section at each age indicated. Laminar boundaries are obtained from an adjacent Nissl-stained section. WM: white matter; s.p.: stratum pyramidale; GL: granule cell layer. Scale bar = 500 μm.

not possible to detect GAT-1 in neuronal somata at the light microscopic level, the Golgi complex of some interneurons displayed sparse immunolabeling at the electron microscopic level [47].

GABA is one of the earliest expressed neurotransmitters in the mammalian cerebral cortex during development. GABA-synthesizing or -containing neurons are present prenatally in rodents and other species [14,16–18,30,33,35,49,52,60–62,64,65]. GABA-accumulating activity is also detected in early developmental stages in the cerebral cortex [14,63]. Additionally, neighboring cells differ considerably in their GABA uptake capability, but strong and light GABA-accumulating cells do not show differences in their positions, orientations, or fine structure [14]. Moreover, it has been suggested that GABA plays a neurotrophic role during development [4,14,20,27,30,37,40,56,60,61,65]. However, little is known about the GABA transport mechanism used by developing GABA-synthesizing neurons. To obtain a better understanding of the role of the GABAergic system in the developing cerebral cortex, it is necessary to characterize the cellular localization of GABA transporter molecules during cortical development. Therefore, we studied the developmental localization of GAT-1 in the neocortex and hippocampal formation of rats from the day of birth to the adult stage with immunocytochemistry.

2. Materials and methods

2.1. Animals

The experiments were performed on male and female albino rats (Sprague-Dawley) at different postnatal ages. The day of birth was referred to as postnatal day 0 (P0) and the time points examined were at P0, P2, P5, P10, P20, P30, P45, P60, and adult stage (around P90). In each of the above groups three or four animals were investigated.

2.2. Tissue preparation

The animals were deeply anesthetized with sodium pentobarbital and perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde and 1% glutaraldehyde in phosphate buffer (PB, pH 7.4). The brains were then removed and postfixed in the perfusion solution for 4–8 h at 4°C, and transferred to cold PBS for several hours to a few days. Brain blocks containing the entire cerebrum and diencephalon were sectioned coronally or horizontally at 50 μm with a vibratome, and alternate sections were collected in PBS and processed for immunocytochemistry and Nissl staining (cresyl violet).

2.3. Immunocytochemistry

Free-floating sections were stained for GAT-1 using 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 blocked 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 a rabbit anti-GAT-1 primary antibody at a concentration of 1:2000 overnight at 4°C and with agitation. After several rinses with PBS, the tissue was further incubated in 1% goat anti-rabbit IgG with 0.5% NGS in PBS for 1 h at room temperature, followed by an incubation in 1% ABC solution for another hour. The immunoreaction products were visualized with 0.05% DAB and 0.005% H_2O_2. Three 10-min rinses with PBS were used between all incubations.

The primary antibody was obtained from Chemicon Inc. (Temecula, CA). Other immunoreactive reagents were obtained from Vector Laboratories. The antibody was tested on the brain tissue at a series of concentrations from 1:500 to 1:5000 and the optimal dilution was 1:1000 to 1:2000. Processing the sections with the primary antibody omitted, or replaced by normal rabbit serum, yielded no specific immunolabeling. In several experiments, brains from two or more age groups were processed at the same time under identical conditions.

To assess the laminar distribution and relative numerical density of the immunolabeled somata, camera lucida drawings of the immunoreactive perikarya in the parietal cortex and hippocampal formation were made at low magnification. The laminar boundaries were obtained from adjacent Nissl sections and were transferred to these maps (Fig. 7).

2.4. Electron microscopic preparation

Pre-embedding immunocytochemistry was performed on vibratome sections for characterizing the ultrastructure of GAT-1-immunoreactive profiles. The sections were first immunostained for GAT-1 as described above, except that Triton X-100 was not used. After immunostaining, selected sections from brains aged at P5, P10, P20, P30 and P45 were post-stained with osmium tetroxide, dehydrated with ethanol and acetone, and flat-embedded in Epon. Thin sections were obtained from neocortical and hippocampal regions, and were stained with uranyl acetate and lead citrate before examination with a Philips CM10 transmission electron microscope.

3. Results

GAT-1 immunolabeling was found in the neocortex and hippocampal formation in all brains examined. The im-

munostaining in the newborns and the 3 oldest groups was solely associated with neuropil, whereas for the other age groups cell bodies were also labeled. In addition, the pattern of the labeled neuropil and the immunoreactivity of the somata exhibited age-dependent changes. Furthermore, the pattern of the GAT-1 immunolabeling did not exhibit any notable areal difference across the rostrocaudal extent of the cerebral cortex and between the two hemispheres of the same brain. Representative photomicrographs in this report were obtained from the parietal cortex and underlying hippocampal formation. The cortical and hippocampal stratification of the developing rat has been described elsewhere [5–7,14,52,62,66].

3.1. Distribution and development of immunoreactive neuropil

3.1.1. Postnatal day 0

At P0, layers V, VIa and VIb were readily identifiable whereas layers II to IV remained undifferentiated within the cortical plate which spanned the area between layers I and V. At this stage, the strongest GAT-1 immunoreactivity was present in layer I, particularly its upper half (Fig. 1A). In the remaining cortical layers, the neuropil immunoreactivity exhibited an inside-out graded staining intensity. Thus, the immunolabeling in layer VIb was light to moderate, whereas the staining in the cortical plate was faint. There was virtually no immunoreactivity deep to the cortex in the immature white matter that included the intermediate and ventricular zones (Fig. 1A).

In the hippocampal formation (Fig. 1B–D), the immunoreactivity was more intense and differentially distributed than that in the neocortex. In Ammon's horn, the density of immunolabeling was moderate in strata oriens, radiatum and lacunosum-moleculare. The stratum pyramidale contained very few immunoreactive puncta (Fig. 1B, C). In the dentate gyrus, the immunolabeling was strong at the upper border of the granule cell layer (i.e., the supragranular band), light within the granule cell layer, and moderate in the hilus (Fig. 1B, D).

3.1.2. Postnatal days 2–5

At P2, immunoreactivity in the neuropil was increased in all cortical layers except layer I, but mainly in layer V to the deep part of the cortical plate, which represented the differentiating layer IV. The prospective layers II and III in the upper cortical plate were faintly stained. The immunoreactivity in layer I was still very strong as compared to the other parts of the developing neocortex. The immunolabeling in layer VIa was slightly lighter than that in V and VIb (Fig. 2A).

At P5, GAT-1 immunoreactivity in the neocortex was increased in layers that had originated from the cortical plate (Fig. 2B). Considerably dense labeling was observed in layer IV and the differentiating layer III. The latter together with the prospective layer II still remained as the cortical plate at this stage. In contrast, the labeling in layers I and VIb was reduced as compared to that at P0 and P2. Thus by P5, the immunolabeling in the neuropil had extended through the entire cortical width, with a peak in layer IV and the greatest staining in layer I (Fig. 2B).

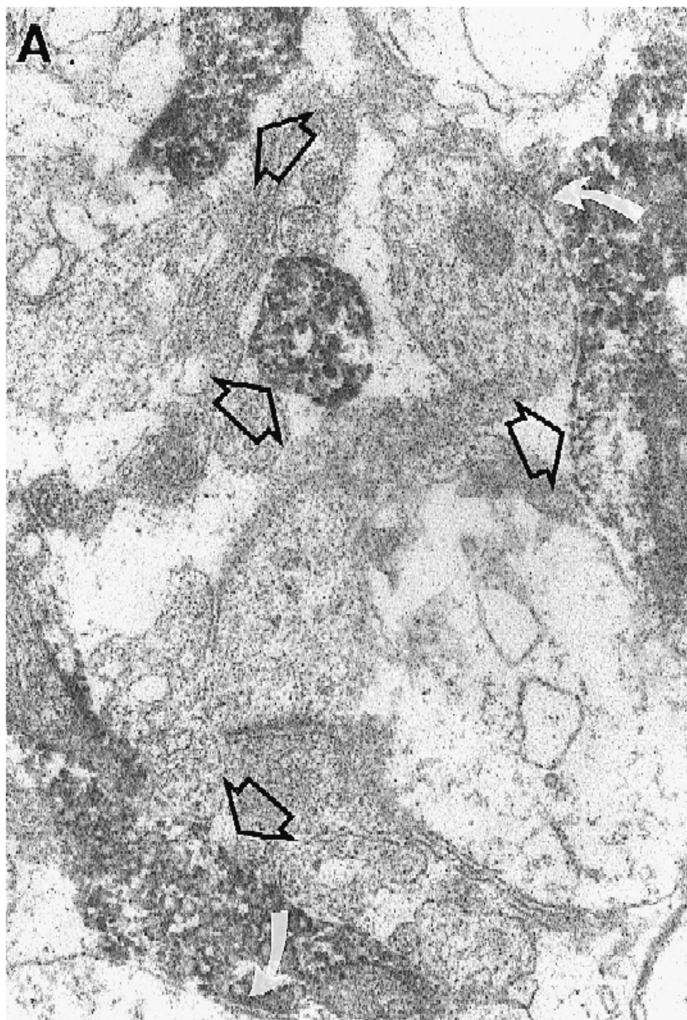
During this same time period, the overall density of the immunoreactive puncta in the hippocampal formation was increased to some extent, as compared to P0. Even though the distribution pattern of GAT-1 immunostaining was the same as that at P0, the immunolabeling was more densely packed in the non-cellular strata, especially the supragranular band in the dentate gyrus (Fig. 2D). The immunoreactive punctate profiles in the pyramidal and granule cell layers were also increased in number, but were not yet associated with, or apposed to, the somata of these layers (Fig. 2C, D).

3.1.3. Postnatal day 10 to adult stage

At P10, the immunoreactivity in the neuropil in layers II to VI, especially II to IV, was increased further as compared to P5. This change together with the dramatic reduction of immunoreactivity in layer I resulted in a homogeneous staining intensity in layers I to IV (Fig. 2B, Fig. 3A). Between P20 and P45, the intensity of the immunoreactivity in punctate structures was continuously increased with a greater increase in the supragranular layers (Fig. 3B–D). Thus, the adult pattern of neuropil staining was reached in the second postnatal month (Fig. 3E).

The overall immunostaining density in the hippocampal formation at P10 was reduced as compared to that at earlier stages. For example, the dense band of immunostaining above the granule cell layer in P0 rats was no longer present (Fig. 1B,D, Fig. 2C,D, Fig. 4A,B). Although the laminar pattern of the immunoreactive puncta at this age and later was basically similar to that at earlier ages, a number of developmental changes was noticed. First, the dot-like immunoreactive profiles were much finer than that seen at P0–P5. Second, the staining intensity above the granule cell layer was reduced from P10 to P45, so that the dark supragranular band seen at earlier stages was no longer apparent (Fig. 4). Third, the immunoreactiv-

Fig. 8. Electron micrographs showing GAT-1-immunoreactive axon terminals (open arrows) in the rat neocortex and hippocampus at P5 (A,B) and P10 (C,D). (A) shows immunolabeled axon terminals in layer I. Two of them form synapses with unlabeled dendritic branches (curved arrows). (B) shows three labeled axon terminals and their possible immature symmetric synapses in the supragranular band (SGB) of the dentate gyrus (arrows). (C) shows labeled axon terminal apposed to another axon and the soma of a granule cell of the dentate gyrus. (D) shows 4 labeled axon terminals in the vicinity of an unlabeled dendrite in the SGB of the dentate gyrus. Scale bar = 0.5 μm.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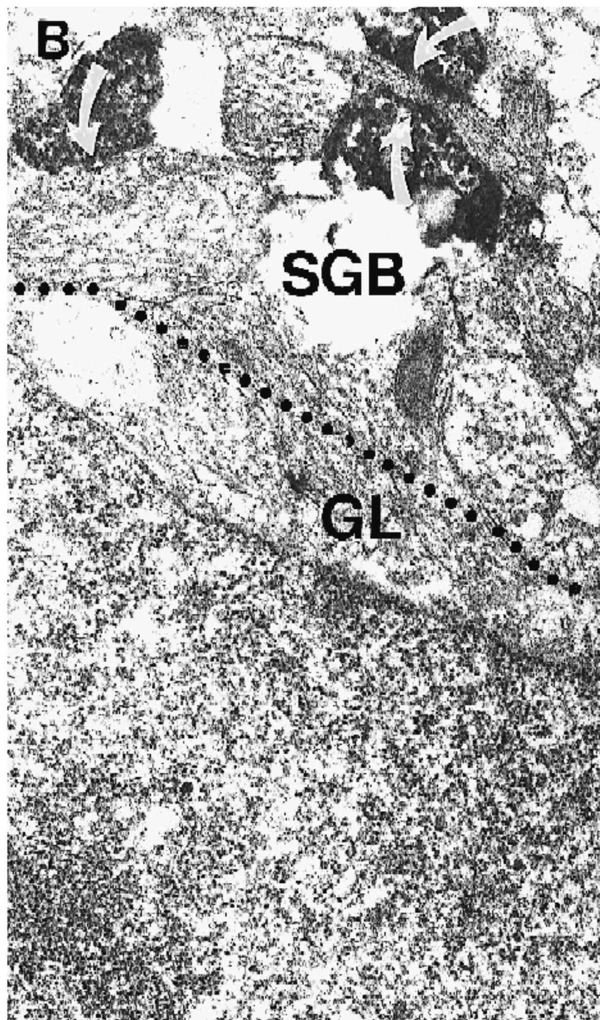
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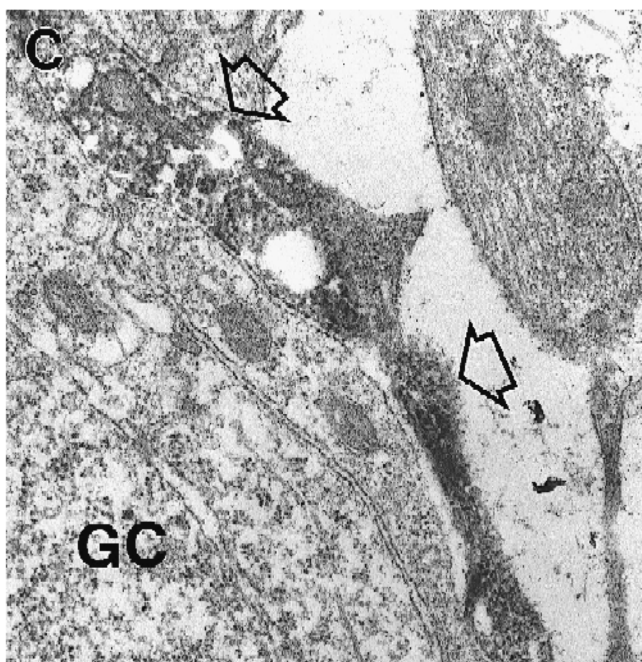
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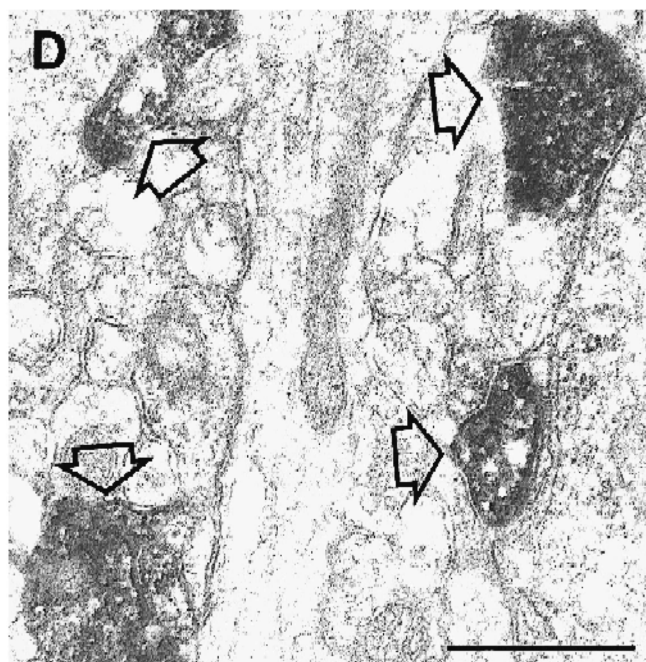
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ity for GAT-1 deep to the pyramidal and granule cell layers was increased with age, and this resulted in an immunoreactive band that appeared at the lower borders of both cellular layers. The immunoreactivity in the subgranular region became the strongest density for the hippocampal formation at P45 (Fig. 4E). Finally, the immunoreactive puncta in the pyramidal and granule cell layers were enriched between the immunonegative cell bodies (Figs. 4 and 6).

3.1.4. Details of the neuropil immunolabeling

At high magnification, the GAT-1 neuropil immunoreactivity exhibited certain changes in their fine distribution and morphology with development. At the youngest 3 ages, the punctate profiles in the neocortex and hippocampal formation were dot-like and largely distributed in a diffuse fashion (Fig. 1C,D, Fig. 2A,C,D, Fig. 5A). After P5, the puncta were increasingly aggregated to align with immunonegative somatic profiles in both the neocortex and hippocampal formation. The immunoreactive puncta were seen to accumulate on the somal and dendritic surfaces of immunonegative neurons. This pattern progressed from the second to fourth postnatal weeks (Figs. 5 and 6). In the P30 and older rats, the most immunoreactive puncta in the neocortex seemed to be preferentially distributed in that they mainly outlined the cortical pyramidal cells as pericellular 'basket' structures (Fig. 5F,G).

Similar to the neocortex, the immunoreactive puncta in the hippocampal formation also showed a developmental association with the somata and primary dendrites of immunonegative pyramidal and granule cells. The intercellular spaces between neighboring pyramidal or granule cells were loaded with immunopositive profiles in rats older than P10, and the pericellular localization of the GAT-1 immunoreactivity in the cellular layers was very distinct by P30 (Figs. 4 and 6).

3.2. GAT-1-immunoreactive somata

A transient expression of GAT-1 in a subpopulation of cell bodies was observed in both the neocortex and hippocampal formation during the first postnatal month. Immunolabeled somata could be detected in the neocortex and hippocampal formation by P5 (Figs. 2, 3 and 7). Subsequently, the number, as well as the immunoreactivity, of the GAT-1-immunoreactive somata increased rapidly, reaching peak values during P10–20. The labeled somata were reduced in number and immunoreactivity by P30 (Fig. 7), and they were not evident at older ages .

3.2.1. Neocortex

The GAT-1-immunopositive somata in the neocortex were found in all cortical layers, including layer I, and in the white matter. The majority of them were localized in layers II–VIb of the cortex, with a slightly denser distribution in the middle layers. This laminar distribution pattern of the immunoreactive somata was present at all ages from P5 to P30 (Fig. 7).

The labeled perikarya in the neocortex were of various sizes and shapes. All GAT-1-immunoreactive somata in the neocortex were non-pyramidal, with small to medium sizes and bipolar, fusiform, bitufted, or multipolar shapes (Fig. 3A–C, Fig. 5A–E). At P5, the great majority of the labeled somata was bipolar in shape. At later ages, immunopositive somata of other shapes were encountered.

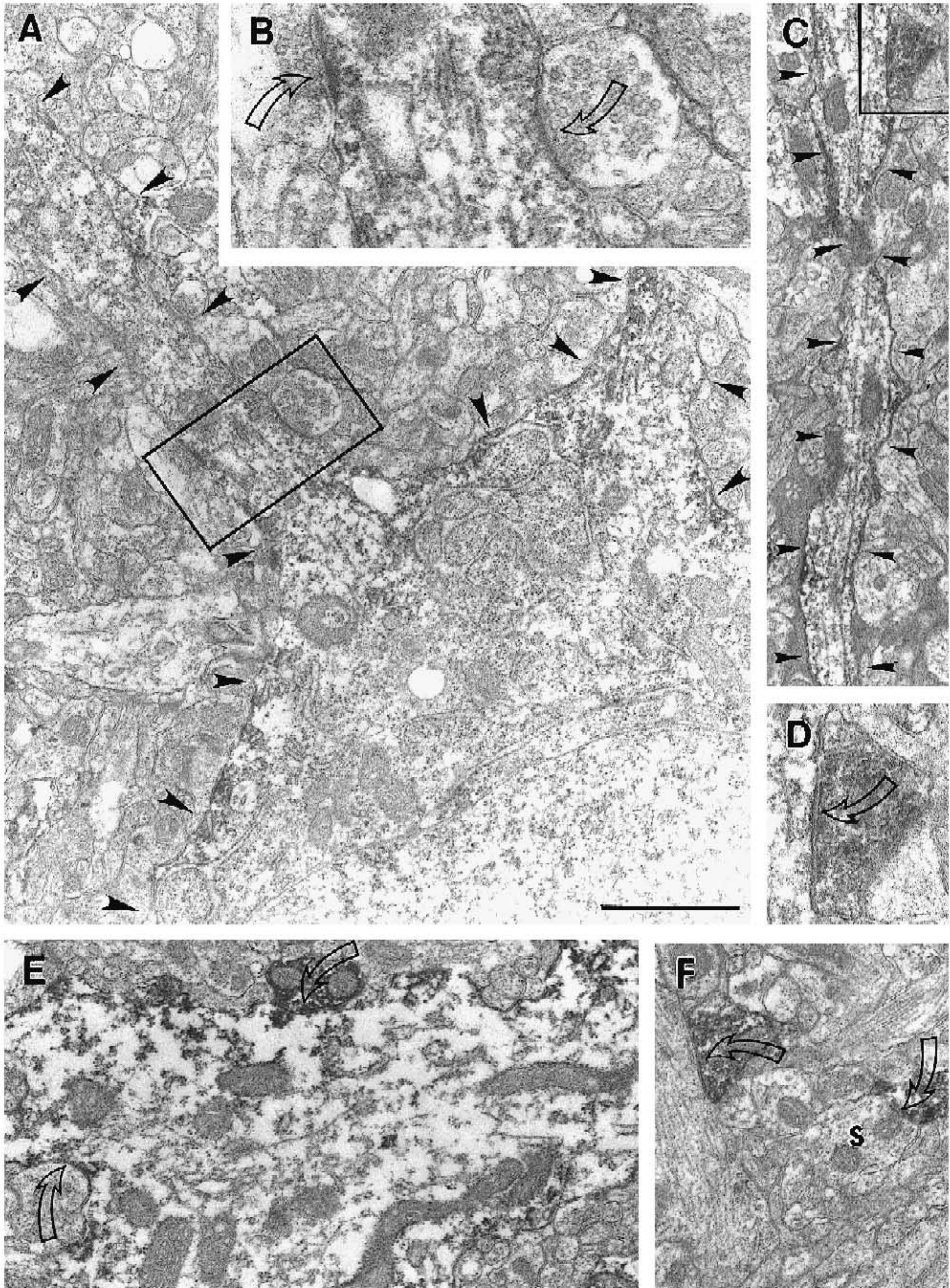
The GAT-1 immunoreactivity in cell bodies was located in the perikaryal cytoplasm, but not the nucleus. At P5, the immunostaining was found in the somata and proximal dendrites. At P10 and P20, the immunolabeling could be traced into the processes for considerable distances (Fig. 5A–E). By P30, the immunoreaction product in the cell body was frequently seen to be localized mainly in the peripheral cytoplasm and in the proximal dendrites (Fig. 3C). As shown below, the electron microscopic preparations did not show a clear association between the immunostaining and any specific intracellular organelle.

To determine whether the reduction in somal staining was due to a lack of GAT-1 in somata from rats at P45 and older ages, sections were immunolabeled with a concentrated primary antibody. Such sections resulted in an elevated neuropil immunoreactivity, but yielded no perikaryal labeling (Fig. 3E).

3.2.2. Hippocampal formation

The GAT-1-immunoreactive cell bodies were encountered in all strata of the hippocampus and the dentate gyrus (Figs. 4 and 7). They showed similar morphological features as those in the neocortex (Figs. 5 and 6). The neurons in strata radiatum, oriens and lacunosum-moleculare in Ammon's horn and the molecular layer of the dentate gyrus were mostly small and round (Figs. 4 and 6). However, the immunopositive somata in the hilus, and those within or at the upper and lower borders of the pyramidal and granule cell layers, were mainly large.

Fig. 9. Electron micrographs of a GAT-1-immunolabeled neuron and its processes obtained from layer IV of the rat neocortex at P20. The distribution of the immunoreaction product is mainly subjacent to the plasmalemma. (A) shows a part of the cell body and two dendrites (indicated with arrowheads). (B) is from the boxed region in (A) and shows two asymmetric synapses (open arrows) on the proximal dendrite formed by unlabeled axon terminals that contain round vesicles. (C) shows a labeled secondary dendrite with a synapse (curved arrow in D) formed by an immunolabeled axon terminal (boxed area) which is enlarged in (D). (E) shows a proximal dendrite of this neuron with a labeled (top center) and an unlabeled (lower left) axon terminals (open arrows). (F) shows immunoreactive axon terminals synapsing on an unlabeled dendritic shaft and spine (s). Scale bar = 1 μm in (A,C,E) and 0.5 μm in (B,D).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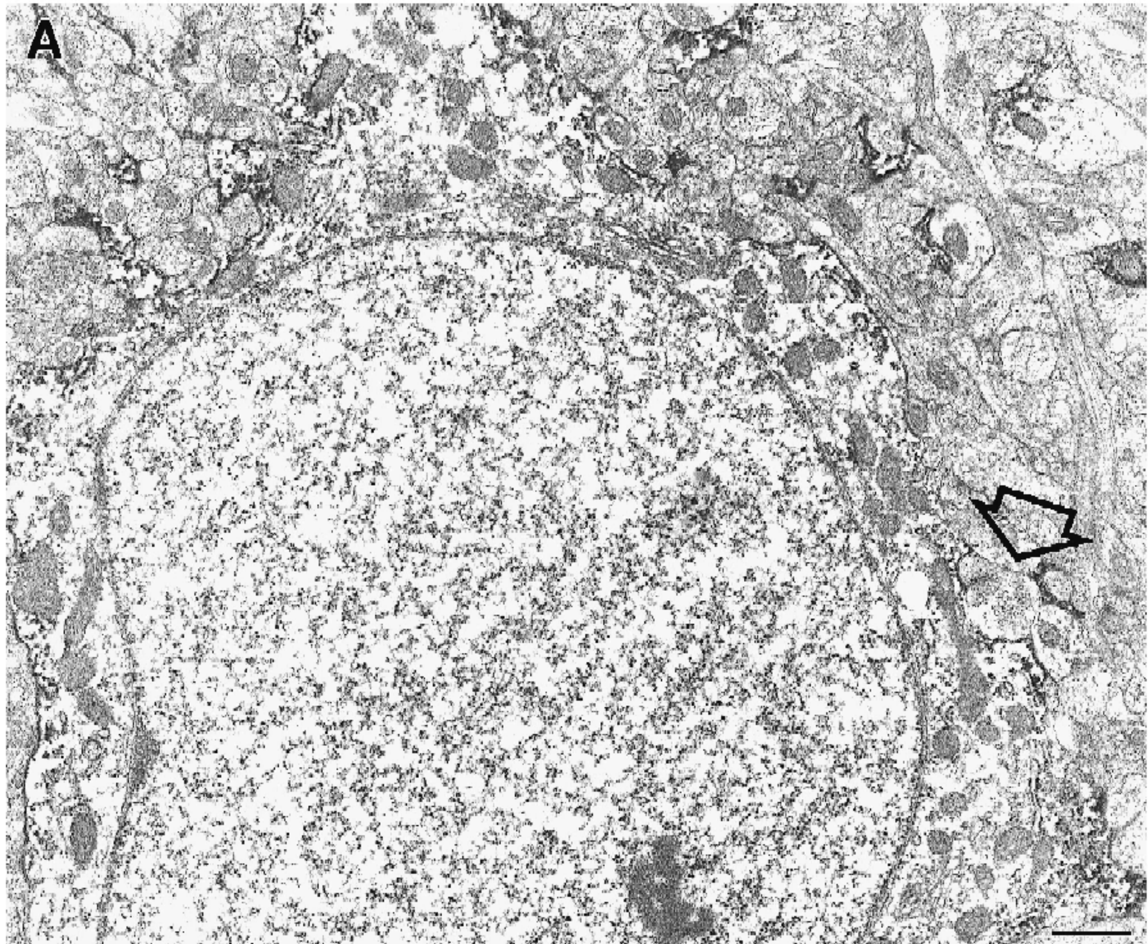
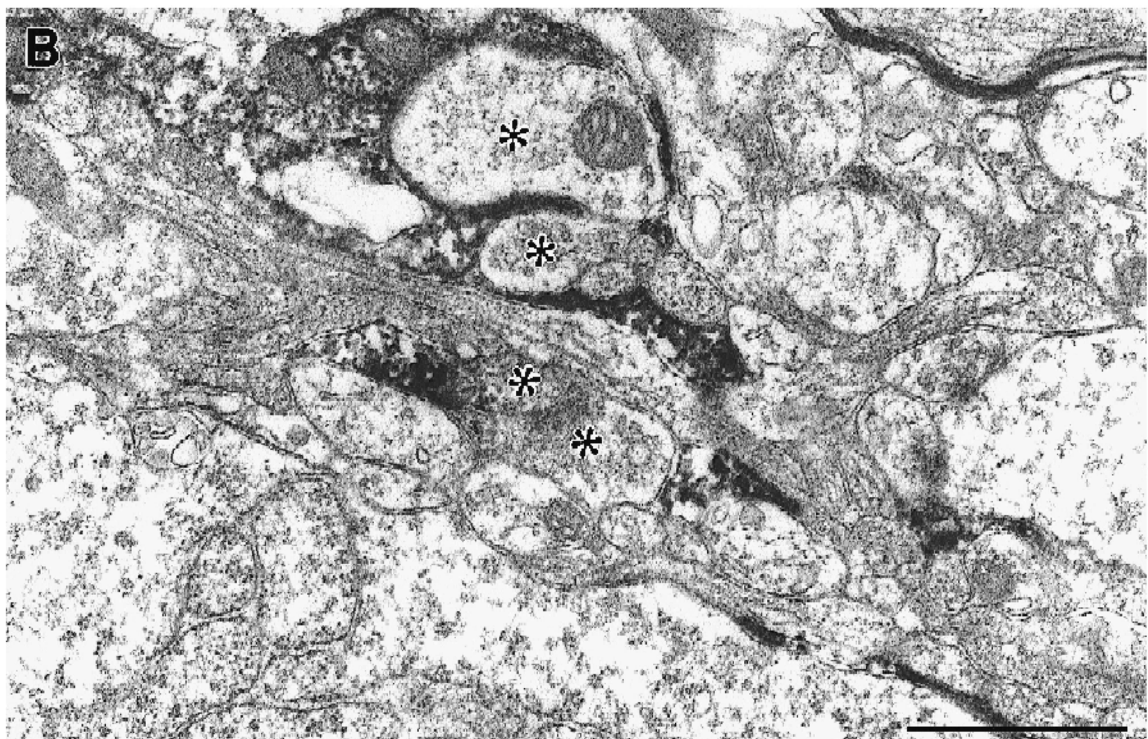

A


B
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Some of them had piriform shapes and were likely pyramidal basket cells, including the inverted pyramidal basket cells (Fig. 6). Other basket-like cells were in the subgranular zone with their long axis oriented parallel to this cell layer (Fig. 4BFig. 6E,F).

3.3. Electron microscopic observations

At the electron microscopic level, GAT-1 immunolabeling was detected in both neurons and glial cells in the developing neocortex and hippocampal formation of the rat (Figs. 8–10). At P5, the dense bands of GAT-1-immunoreactive puncta in layer I of the neocortex and the supragranular band of the dentate gyrus from light microscopy were found to be composed of mostly GAT-1-immunopositive axon terminals (Fig. 8A,B). Some of these axon terminals contained immunolabeled vesicles and formed symmetric synapses with unlabeled dendritic processes. At P10 and P20, GAT-1 immunoreactivity in neuronal somata and astrocytes was distinctly identified (Figs. 9 and 10). The GAT-1 immunolabeling in somata was present in the peripheral cytoplasm (Fig. 9A), and that in dendrites was detected throughout their cytoplasm (Fig. 9A–E). In both sites the immunoreactivity was more concentrated in sublemmal regions and subjacent to postsynaptic membranes (Fig. 9C–E). GAT-1-immunoreactive neurons were postsynaptic to axon terminals that were immunopositive and immunonegative. The immunonegative axon terminals often had round vesicles, and appeared to have thickened postsynaptic membranes (Fig. 9A,B,E). In contrast, labeled axon terminals contained oval or round vesicles and formed synapses with immunonegative and immunopositive dendrites or somata. These synapses seemed to be symmetric as no pronounced postsynaptic thickness was detected (Fig. 9C–F).

GAT-1 immunolabeling in astrocytes was observed in both their somata and processes (Fig. 10). The immunoreactive somata had little cytoplasm, and displayed thin and irregularly shaped processes that usually extended between neuronal profiles. Often, these labeled glial processes surrounded axon terminals, and sometimes enveloped both the pre- and postsynaptic profiles (Fig. 10A,B).

4. Discussion

The present study examined the postnatal development of GAT-1 immunoreactivity in the neocortex and hippocampal formation of the rat. One of the major findings of this study is that GAT-1 is strongly expressed in cortical and hippocampal neuronal somata during development. Another interesting observation is that the immunostaining for GAT-1 in layers I and VIb of the neocortex and the supragranular band in the dentate gyrus displays a dramatic developmental regression. In the following sections, we will first characterize the cellular nature of the immunopositive somata, then compare our findings of the development of GAT-1-immunoreactive somata and puncta with previous observations of other GABAergic parameters, and finally discuss the possible role of GAT-1 in the developing rat cerebral cortex.

4.1. Somal immunolabeling for GAT-1 in immature cortex

The most significant finding of the present study is the transient presence of GAT-1 immunoreactivity in the cell bodies and processes of a subpopulation of cortical and hippocampal neurons during a restricted time period. The labeled cell bodies are found mainly after the first postnatal week and up to the end of the first month in both the neocortex and hippocampal formation of the rat.

These GAT-1-immunoreactive somata are most likely GABAergic interneurons. This suggestion is supported by the following facts. First, the morphology of the GAT-1 positive somata is typical of non-pyramidal neurons, and not cortical principal neurons. The majority of the labeled somata in the neocortex and hippocampus are small to medium in size. The somal shapes are round, oval, bipolar, bitufted and multipolar. Cortical neurons with these morphological features have been shown to be interneurons, particularly GABAergic neurons, as determined with the localization of GAD and GABA, and other markers, such as calcium-binding proteins, neuropeptides and NADPH-diaphorase that colocalize with GABA [11,18,22,23,30,44,46,51,52,55,65–69]. In the hippocampus, the GAT-1 positive somata within and near the pyramidal and granule cell layers appear to be GABAergic basket cells, including the pyramidal basket neurons, according to their morphology and distribution [45,46,51,53,55]. Second, the numerical density and laminar distribution pattern of GAT-1 positive somata in the neocortex and hippocampal formation at P10–30 are strikingly similar to that of GAD and GABA-immunoreactive cells at the corresponding developmental stages [14,18,52,62]. Third, neurons expressing GAT-1 in adult rats were identified as GABAergic interneurons using in situ hybridization [43] or electron microscopic immunocytochemical methods [47]. Therefore, the data indicate that GAT-1 is transiently expressed in somata of GABAergic cortical neurons at detectable levels in light microscopic preparations from early developmental stages, and that for some reason, GAT-1, is not present in such high amounts

Fig. 10. Electron micrographs of GAT-1 immunoreactivity in an astrocyte from a P20 rat. (A) shows a labeled astroglial soma and its processes in the adjacent neuropil. (B) shows immunolabeled glial processes. Note that the astroglial processes tend to surround axon terminals and their postsynaptic targets (arrow in A and asterisks in B). Scale bar = 1 μm.

in the somata following the maturation of these interneurons.

The present study confirms previous observations of the presence of the 'neuronal' GABA transporter, GAT-1, in astrocytes in the rat neocortex and hippocampus [38,47]. Our results showed that the immunolabeling in astroglia of the immature rat cerebral cortex is distinctly detectable in their somata at the electron microscopic level. In contrast, GAT-1 immunolabeling is mainly present in astrocytic processes in the adult rat [38,47]. As with the somata of interneurons, GAT-1 in astrocytic somata may indicate that they are involved in the regulation of GABA's action in a different way during development from that in the adult (see the last section of Discussion).

4.2. Developmental coincidence of GAT-1, GAD and GABA immunoreactivities

GAT-1 immunoreactivity is differentially developed in the neocortical and hippocampal layers. In addition to the transient somatic labeling mentioned above, there is an impressive developmental downregulation of the neuropil immunoreactivity in layer I and VIb of the neocortex and the supragranular band of the dentate gyrus. In contrast, the neuropil immunoreactivity in the cortical plate and its derivatives (layers II to VIa) is increased remarkably during the first 2–3 postnatal weeks following an inside-out order, and an adult pattern with the greatest labeling localized in layers II to IV is established during the second postnatal month. Similarly, the neuropil immunoreactivity in most strata of the hippocampal formation, especially the cellular layers and the hilus, is increased with age until P30–45 when an adult distribution is achieved.

GAD- and GABA-immunoreactive neuronal elements can be detected in the rodent cerebral cortex several days before birth [16–18,30,60,62]. The prenatal GABAergic neurons and processes are present early in the marginal zone and subplate (prospective layers I and VIb), which are the earliest generated cortical elements. GABAergic terminals in these regions may also originate from subcortical structures [7,16,30,36,50,60,65]. The GABA immunostaining in these areas regresses during the late prenatal and early postnatal stages. GABA-immunoreactive somata and puncta in the derivatives of the cortical plate develop later. They appear in the deep cortical layers at perinatal stages, and proceed from deep to superficial layers during the first several postnatal weeks, and gain an adult-like laminar pattern with the highest density in layers II–IV by the end of the first postnatal month [35,62].

GABAergic neuronal somata and puncta in the rat hippocampus are detectable at P0[52]. GABA- and GAD-immunoreactive neurons in the rat hippocampus and dentate gyrus appear mostly from P5 to P16 (70% adult value), and they gain 90% of the adult number by P18, and reach adult levels by P24 [52].

The postnatal development of GAT-1 immunoreactivity in both neocortical and hippocampal regions of the rat coincides well with that of GAD and GABA immunoreactivity. First, the regressive course of GAT-1 immunoreactivity in layers I and VIb is similar to that of GABA and GAD immunolabelings. Second, the timing and pattern of the laminar differentiation of GAT-1 immunoreactivity is correlated with that of GABA or GAD immunostaining. Third, the developmental changes in the detailed distribution of the puncta from a largely homogeneous arrangement to associate preferentially with pericellular formations of the principal neurons is the same for the three markers [16,18,30,35,49,52,62]. Thus, the expressions of GABA and its transporter, GAT-1, are tightly linked during development in the neocortex and hippocampal formation, indicating that GAT-1 expression during development relates to the functional maturation of the GABAergic system.

4.3. Parallelism of GAT-1 immunoreactivity with GAT-1 mRNA expression and GABA uptake ability

The GAT-1 mRNA level in the rat cerebral cortex is low at birth (25% of adult value), reaches the adult level by the end of the first postnatal week, and achieves a plateau 25–40% above adult levels between P10 and P30 [70]. GABA uptake is also well below adult levels at birth, then reaches values that are 30–60% above adult levels over the same time period [17,64]. Furthermore, endogenous GABA concentrations and GAD activity in tissue from the developing rat cerebral cortex are low at birth and exceed adult values before adult levels are achieved [17].

In the present study, the overall immunoreactivity in the neuropil of the developing rat cerebral cortex did not display a higher staining intensity between P10–30 than at later ages, including the adult. However, a large number of neuronal somata and dendrites were immunostained for GAT-1 during this period, and this labeling was subsequently lost. It is likely that this labeling of GAT-1 between P5–30 could be the basis for the greater than adult levels of both GAT-1 mRNA expression and GABA accumulation ([17,64,70], also see below).

4.4. Functional implications

Synaptogenesis of cortical neurons, including GABAergic interneurons, has been studied in the rat [2,8,10,41,53,54,63]. Gray's type II, or symmetric, synapses are formed by the axon terminals of non-pyramidal, especially GABAergic, neurons. Electron microscopic studies in the rat neocortex showed that type II synapses are fairly sparse at P6, increase dramatically in the second and third postnatal weeks, and then decline markedly between P20 and P90. Type II synapses are mainly distributed in the basal layers at P6, and concentrated in layer IV by P8. The adult-like pattern of distribution of type II synapses is achieved by the end of the second postnatal week, with the

majority of the synapses being present in layers II–IV [2,8].

Previous studies have shown that most GABAergic parameters share a developmental sequence with synaptogenesis in the mammalian cerebral cortex, though the content or immunoreactivities of GAD, GABA, GABA receptors and transporters all reach considerable levels before type II synapses are formed, possibly because GABAergic axons and varicosities develop before symmetric synapses [2,8,10,14,30,60–65,70]. It was suggested that GABA in immature neurons plays a neurotrophic role or acts as an excitatory neurotransmitter, rather than an inhibitory neurotransmitter as in the adult [3,12–14,20,27,30,33,56,60–62,65]. This notion is supported by recent in vitro observations made for GABAergic neurons, and by the fact that GABA may play an excitatory role in neurons under traumatic conditions [4,37,39,40,56,57,59]. The present study indicates that GAT-1 expression in rat neocortex also proceeds in the same spatial order of the synaptogenesis of cortical neurons, but in a slightly advanced time span. An early established GABA transporter system in the cerebral cortex may help to define or guide GABA's role for neuronal morphogenesis. In fact, the formation of symmetric synapses may depend on GABA-mediated modulation of the development of postsynaptic receptors [30]. It is possible that GAT-1 might play a role in determining the prospective synaptic sites, and that in turn may help to specify the formation and activation of synapses.

A relatively mature GABA uptake system in layers I and VIb of the neocortex and the supragranular band of the dentate gyrus at early developmental stages is particularly of interest. In the mammalian neocortex, GABAergic neurons with a morphology resembling Cajal-Retzius cells in layer I are observed at early fetal stages before neurons in the cortical plate differentiate [18,30,60,65]. These layer I neurons also express calcium binding proteins transiently [11,24,69]. In the hippocampal dentate gyrus, the GABAergic axon terminals of basket cells and their synapses are concentrated in a supragranular band during early development [52–54]. The activation of GABA receptors induces a depolarization associated with an increase of intracellular calcium ions during early development and in traumatic mature neurons [4,12,13,20,40,57]. Calcium is critical for morphogenesis, particularly neurite elongation and neuronal migration [34,40]. Thus, the early GABAergic systems in cortical layers I and VIb and the supragranular band of the dentate gyrus may serve an inductive function for the structural maturation of principal neurons in the neocortex and the hippocampus. In an alternative way, the activation of the early GABAergic synapses in layer I and the supragranular band may influence the maturation process of the cortical principal neurons. For example, the synaptic activity of GABAergic axons in layer I might prevent the overgrowth of apical dendrites of pyramidal cells from lower layers [7,36,65,71]. Furthermore, the dense GABAergic axonal plexus in the supragranular band may act as a structural net or barrier to prevent the outward migration of newly generated granule cells beyond this band. In this way, the granule cells that are added to this developing layer remain on the hilar side of the first established row of granule cells [5,6,52–54], in contrast to that in the neocortex where neurons destined for the more superficial layers penetrate through the previously established layers [7,36].

The biological significance of the transient somatic labeling of GAT-1 in interneurons is unknown. However, the electron microscopic localization of GAT-1 immunoreaction product provides a plausible reason for the transient expression of GAT-1 in somata and dendrites. At both of these sites in GAT-1-immunolabeled neurons, the immunolabeling was present subjacent to the plasmalemma. This subcellular localization would indicate that GAT-1 in the GABAergic interneurons of the neocortex and hippocampal formation is involved in the translemmal transportation of GABA throughout great regions of their surfaces. This process would thus enhance the ability of GABA neurons to recycle GABA. On the other hand, since the labeling was not apparently associated with the Golgi complex and its vesicles as it is in the adult [47], the presence of GAT-1 in somata and dendrites may not be for its vesicular transport to axon terminals. In fact, even though GAT-1 is thought to remove GABA from the extracellular space, other data indicate that GAT-1 can expel GABA from axon terminals in a calcium independent, non-vesicular release mechanism [1,3,28,31]. Thus, GAT-1 in sublemmal regions of the cell bodies and dendrites is perhaps mainly involved in releasing GABA from somal and dendritic membranes through this non-vesicular mechanism during early development. As several studies suggest a neurotrophic role of GABA during development [3,12–14,20,27,30,33,56,60–62,65], GABAergic neurons during development could excrete GABA from their soma and dendrites, and GABA can widely affect the developing cortical neurons. Therefore, the transient expression of GAT-1 in somata and dendrites may be one of the most important mechanisms for GABAergic neurons to play a unique morphogenic role during development. Studies of neonatal rat cerebral cortex need to be made to determine whether the role of the transiently expressed GAT-1 in somata and dendrites is for uptake *or* release of GABA.

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