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Embryonic and Postnatal Expression of Four Gamma-Aminobutyric Acid Transporter mRNAs in the Mouse Brain and Leptomeninges

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ABSTRACT

The distribution of gamma-aminobutyric acid (GABA) transporter mRNAs (mGATs) was studied in mouse brain during embryonic and postnatal development using in situ hybridization with radiolabeled oligonucleotide probes. Mouse GATs 1 and 4 were present in the ventricular and subventricular zones of the lateral ventricle from gestational day 13. During postnatal development, mGAT1 mRNA was distributed diffusely throughout the brain and spinal cord, with the highest expression present in the olfactory bulbs, hippocampus, and cerebellar cortex. The mGAT4 message was densely distributed throughout the central nervous system during postnatal week 1; however, the hybridization signal in the cerebral cortex and hippocampus decreased during postnatal weeks 2 and 3, and in adults, mGAT4 labeling was restricted largely to the olfactory bulbs, midbrain, deep cerebellar nuclei, medulla, and spinal cord. Mouse GAT2 mRNA was expressed only in proliferating and migrating cerebellar granule cells, whereas mGAT3 mRNA was absent from the brain and spinal cord throughout development. Each of the four mGATs was present to some degree in the leptomeninges. The expression of mGATs 2 and 3 was almost entirely restricted to the pia-arachnoid, whereas mGATs 1 and 4 were present only in specific regions of the membrane. Although mGATs 1 and 4 may subserve the classical purpose of terminating inhibitory GABAergic transmission through neuronal and glial uptake mechanisms, GABA transporters in the pia-arachnoid may help to regulate the amount of GABA available to proliferating and migrating neurons at the sub-pial surface during perinatal development.

Indexing terms: GABA, pia-arachnoid, cerebellum, in situ hybridization, development

The synaptic action of gamma-aminobutyric acid (GABA) is terminated by high affinity re-uptake transporters. These GABA transporters (GATs) are an abundant and pharmacologically important (Iversen and Bloom, 1972; Iversen and Kelly, 1975) class of plasma membrane proteins that, in the adult central nervous system (CNS), accumulate GABA against a concentration gradient in a Na^+ (Iversen and Neal, 1968) and Cl^- (Kanner, 1978) dependent manner, thus translocating GABA from the synaptic cleft into presynaptic terminals and glia. The GATs belong to a family of Na^+/Cl^- coupled transporter proteins characterized by the presence of 12 transmembrane domains, short N and C termini on the cytoplasmic surface, and a large, potentially glycosylated, extracellular loop between transmembrane segments III and IV (Nelson and Lill, 1994). Other members of this family include the proline, glycine, taurine, betaine, dopamine, noradrenaline, and serotonin transporters (Uhl and Hartig, 1992). Pharmacologic characterization of GABA transporters has determined their presence in both neuronal and glial cells (reviewed by Kanner and Kleinberger-Doron, 1994). In situ hybridization (Clark et al., 1992; Rattray and Priestly, 1993; Swan et al., 1994; Durkin et al., 1995) and immunocytochemical (Radian et al., 1990; Brecha and Wightman, 1994; Ikegaki et al., 1994; Jursky et al., 1994; Itouje et al., 1996) studies suggest both neuronal and nonneuronal expression patterns. Although four different GATs (mGATs 1–4) have been cloned from mouse cDNA libraries (Liu et al., 1992, 1993; Lopez-Corcuera et al., 1992), only three (GAT1/GAT-A, GAT2, and GAT3/GAT-B) have been cloned from rat libraries (Guastella et al., 1990; Borden et al., 1992;

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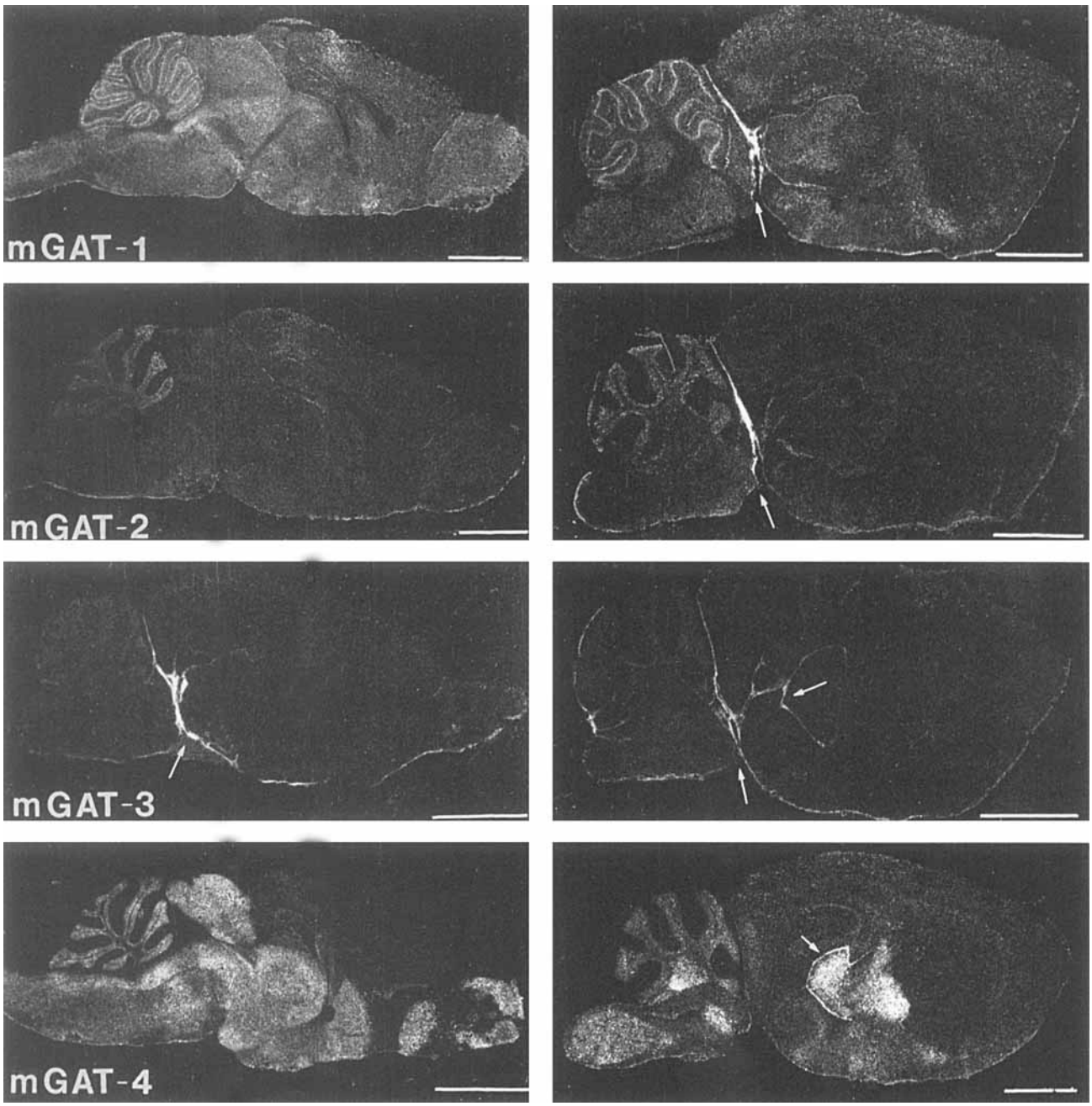

Fig. 1. The expression patterns of the four murine gamma-aminobutyric acid (GABA) transporters (mGAT 1–4) in the central nervous system of adult (P60) C57BL/6J mice. Darkfield autoradiographs of medial (left column) and lateral (right column) sagittal sections. Small arrows indicate labeling in the pia-arachnoid membrane. Scale bars = 2 mm.

Clark et al., 1992). Mouse GAT1 is most similar to rat GAT1 (96% homology), mouse GAT3 to rat GAT2 (95% homology), and mouse GAT4 to rat GAT3 (96% homology); mouse GAT2 resembles the dog betaine transporter (Yamauchi et al., 1992), with 84% homology.

GABA (Lauder et al., 1986), $GABA_A$ receptors (Laurie et al., 1992; Fritschy et al., 1994; Chang et al., 1995), and GABA accumulating (Chronwall and Wolff, 1980; Hatten et al., 1984) and releasing (Taylor and Gordon-Weeks, 1989, 1991) mechanisms are present in the perinatal brain well before synapses are formed. During this time, rather than inhibiting neurons as in the adult, GABA depolarizes immature neurons (Cherubini et al., 1991) and may act as a neurotrophic (Meier et al., 1991; Wolff et al., 1993) and chemokinetic (Behar et al., 1994) agent. The transition from embryonic to adult GABAergic function may be mediated, at least in part, by concomitant switches in subunit composition of $GABA_A$ receptors (Laurie et al., 1992; Fritschy et al., 1994; Chang et al., 1995). Although GABA transporters have been well characterized in the

TABLE 1. Regions Showing the Highest mGAT Expression in the Perinatal and Adult Brain.[1]

CNS region	Perinatal	Adult
mGAT-1		
VZ of ventricles III and IV	+++	–
ganglionic eminence (lat. and med.)	+++	–
SVZ of the lateral ventricle	+++	–
olfactory bulb	+++	+
cortex (layer 1)	+	++
hippocampus and dentate gyrus	++	++
thalamus	+	++
basal forebrain	+	++
hypothalamus	+	++
striatum	+	++
substantia nigra	+	++
vestibular nuclei	+	++
pontine nuclei	+	++
cerebellar Purkinje cell region	++	+++
ventral spinal cord	+++	+
pia-arachnoid membrane	+++	+++
mGAT-2		
cerebellar egl	++	–
cerebellar igl	++	+
pia-arachnoid membrane	+++	++
mGAT-3		
pia-arachnoid membrane	+++	++
mGAT-4		
septal neuroepithelium	+++	–
SVZ of the lateral ventricle	+++	–
olfactory bulbs	+++	++
cortex	++	–
hippocampus and dentate gyrus	+++	+
pontine nuclei	++	+++
vestibular nuclei	++	+++
hypoglossal nuclei	++	+++
dorsal and ventral cochlear nuclei	++	+++
facial nuclei	++	+++
solitary nuclei	++	+++
inferior and superior colliculi	++	+++
inferior olivary nuclei	++	++
cerebellar egl	++	–
cerebellar igl	+	++
cerebellar nuclei	+++	+
spinal cord	+++	+++
pia-arachnoid membrane	+++	+

[1]Subjective grain density values in each region are indicated by +, with 3 being the highest, 2 intermediate, and 1 low. Grain densities may be compared only within probes. egl, external germinal layer; igl, internal granular layer; SVZ, subventricular zone; VZ, ventricular zone.

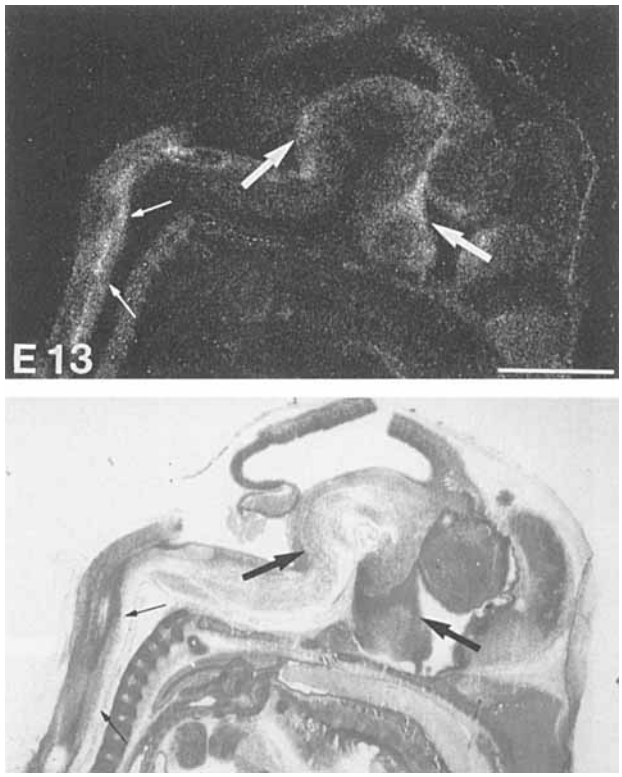

Fig. 2. Darkfield autoradiograph and corresponding cresyl violet-stained section showing mGAT1 expression in the embryonic day (E)13 brain and spinal cord. Large arrows indicate increased grain density in proliferative zones adjacent to the third and fourth ventricles; small arrows indicate labeling in the ventral spinal cord. Scale bar = 2 mm.

adult rodent central nervous system (CNS), and polymerase chain reaction studies have shown that GAT1 mRNA is present in the ventral mesencephalon from embryonic day (E)12 (Perrone-Capano et al., 1994), little is known about the developmental expression of GABA transporters in specific brain regions. In the present study, we used in situ hybridization with ^{35}S-labeled oligonucleotide probes to examine regional changes in the expression of mGAT mRNAs in the late embryonic and postnatal mouse brain.

METHODS

Oligonucleotide probe selection

The mGAT cDNA sequences were retrieved from GenBank (accession numbers: mGAT1, M92378; mGAT2, M97632; mGAT3, L04662; mGAT4, L04663). The cDNA sequence was analyzed for candidate antisense oligonucleotide probes with the aid of Oligo (version 4.0, National Biosciences, Plymouth, MN) and MacDNASIS Pro DNA and protein sequence analysis system (version 1.0, Hitachi Software Engineering, Brisbane, CA). Final candidates were chosen based on the following criteria: low energy or no 3' terminal dimers, low energy or no hairpins, low energy dimers with free 3' end, a roughly equal percentage of G+C and A+T, homogenous distribution of nucleotides, total length of 42–48 bp and low homology to related sequences. Oligonucleotides were synthesized by Operon Technologies (Alameda, CA). The probe sequences were as follows: GAT1: 5'-TGATGACGATGTAGTAGATGTTCAGCCAGAAGGACAGCACAG-3' (nt 530-571); GAT2: 5'-TCTGGCCGATGAGTGATTCAGAAAGTCCACGCAGTGCTCTGT-3' (nt 544-585);GAT3:5'-AGGTGGCATTCTCGGAGGTCACATTCATGGAATCATTGGCCTTCT-3' (nt 530-574);GAT4: 5'-AGTCCGATGATCTCTCCAGCTACGCTCAACACGAACTCCACC-3' (nt 191-232).

Adjacent brain sections, hybridized with sense probes, were used as controls for nonspecific hybridization.

Probe preparation

The oligonucleotides were purified through NAP-10 columns (Pharmacia, Piscataway, NJ). The resulting samples were dried under vacuum, redissolved in 10 μl diethylpyrocarbonate (DEPC)-treated H_2O, and assayed for nucleotide content (1.0 OD at 260 nm ≈ 33 μg/ml). The purified oligonucleotide was 3' end labeled with [^{35}S]-α-dATP (Amersham, Arlington Heights, IL) at an oligo to dATP ratio of 1:10. The oligonucleotide and [^{35}S]-α-dATP were incubated at 37°C for 60 minutes in a buffer consisting of 0.2 mol/L potassium cacodylate, 25 mmol/L Tris-HCl, 125 μg bovine serum albumin, and 5 mmol/L $CoCl_2$. The reaction was catalyzed with 37.5 units of terminal transferase (Boehringer, Mannheim, Germany) and took place in a final volume of 50 μl. The labeling reaction mixture was purified through a Nensorb-20 column (Dupont/NEN, Boston, MA). Specific activity of the oligonucleotide probes was 0.8–1.2 ×

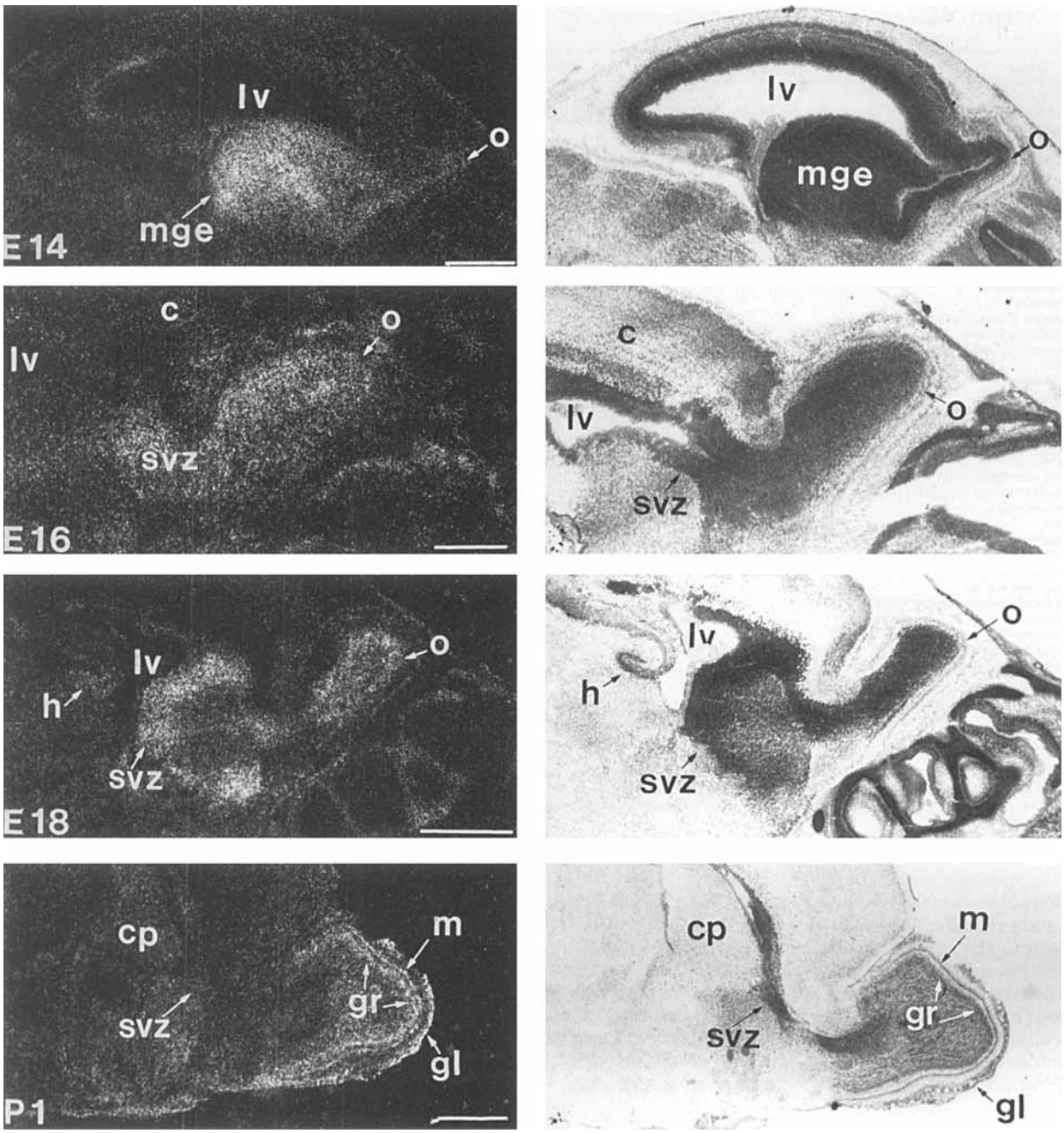

Fig. 3. Corresponding darkfield autoradiographs (left column) and brightfield counterstained sections (right column) through the perinatal C57BL/6J mouse brain. Note high levels of mGAT1 mRNA in the medial ganglionic eminence (mge), a proliferative region adjacent to the lateral ventricle (lv). c, cerebral cortex; cp, caudate putamen; h, hippocampus; o, olfactory bulb; svz, subventricular zone of the rhinencephalon. Within the olfactory bulb: gr, granular layer; m, mitral cell layer; gl, glomerular layer. Scale bars = 500 μm in E14, 16, P1, 1 mm in E18.

10^9 dpm/μg. The GAD67 probe (a gift from Dr. Allan Tobin, UCLA, Los Angeles) was prepared as described previously (Zdilar et al., 1992).

In situ hybridization

C57BL/6J mice were bred from stocks originating at Jackson Laboratories (Bar Harbor, ME). Developing animals (E13 to postnatal day (P)60) were decapitated under anesthesia (Metofane, Pitman-Moore, Mundelein). Brains were removed rapidly, frozen in powdered dry ice, and stored at −70°C. Sections, 20-μm thick, were thaw-mounted onto 3 × subbed (300 bloom gelatin and chrome alum) slides and stored at −70°C. Before hybridization, sections were fixed for 10 minutes in 4% paraformaldehyde in phosphate-buffered saline (PBS, 0.01 mol/L, pH 7.4) and washed (2 × 5 minutes) in PBS. Sections were then acetylated (0.25% acetic anhydride in 0.1 mol/L saline triethanolamine hydrochloride [TEA], pH 8.0) for 10 min-

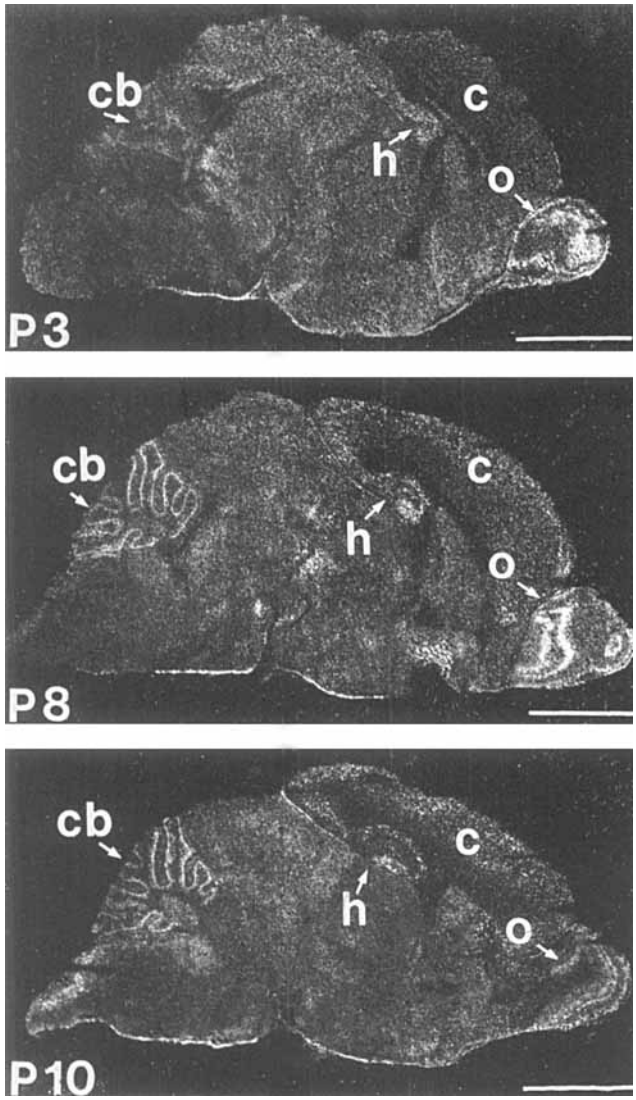

Fig. 4. Darkfield autoradiographs of sagittal sections through the postnatal mouse brain showing mGAT1 expression at postnatal day (P)3, P8, and P10. c, cerebral cortex; cb, cerebellum; h, hippocampus; o, olfactory bulb. Scale bars = 2 mm.

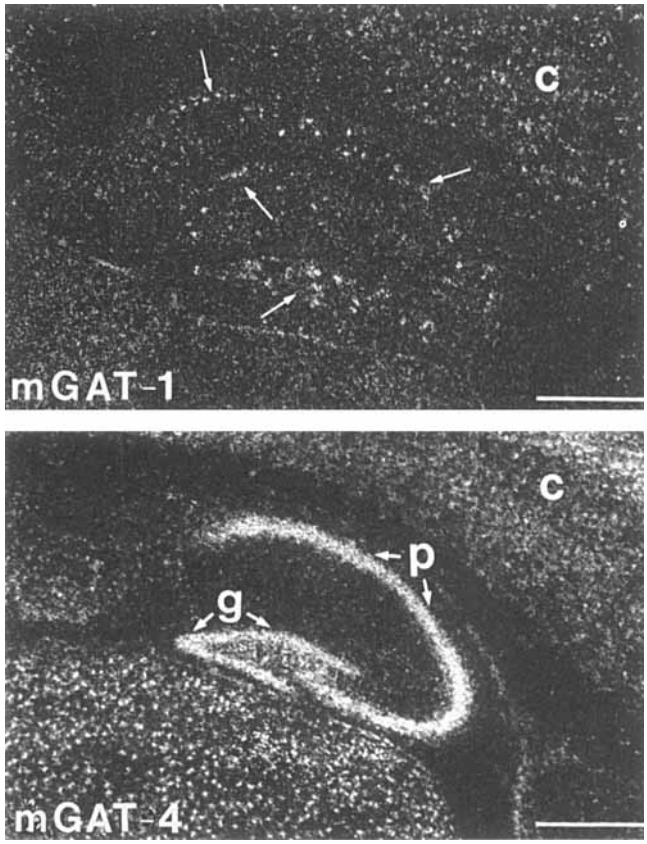

Fig. 5. Mouse GAT1 and mGAT4 mRNA expression in the hippocampus and dentate gyrus at P10. Small arrows in the top panel show punctate clusters of the mGAT1 message scattered throughout both structures. In the lower panel, high levels of mGAT4 expression are present in the hippocampal pyramidal layer (p) and granule cell layer of the dentate gyrus (g). c, cerebral cortex. Scale bars = 500 μm.

utes at room temperature, washed (2 × 2 minutes) in 2 × saline sodium citrate (SSC, 0.03 mol/L citric acid, trisodium salt in 1.8% NaCl), dehydrated (1 minute each) through 70%, 80%, 90%, and 100% ethanol, delipidated in 100% chloroform (1 minute), and rinsed in 100% ethanol.

Hybridization was conducted as follows. Each slide was covered with 100 μl of hybridization buffer (50% formamide, 4 × SSC, 500 μg/ml salmon sperm DNA, 250 μg/ml tRNA, 1 × Denhardt's, 100 mmol/L dithiothreitol and 10% dextran, containing 3.0×10^7 dpm/ml oligoprobe), covered with parafilm, and incubated for 20 hours at 42°C. The parafilm was removed in 1 × SSC, and the sections were washed in 2 × SSC at room temperature for 10 minutes. Sections were then washed at increasing stringency as follows: 2 × SSC (10 minutes), 1 × SSC (10 minutes), 0.5 × SSC (10 minutes), 0.25 × SSC (10 minutes), 0.125 × SSC (10 minutes), and 0.125 × SSC (60 minutes) at a washing temperature of 60°C. Following a final wash in 0.125 × SSC (10 minutes) at room temperature, the sections were dehydrated (1 minute each) through 70%, 80%, 90%, and 100% ethanol. All washing solutions contained 10 mmol/L 2-mercaptoethanol, to prevent nonspecific binding of the oligoprobe. The nonspecific hybridization signal was determined by exposing adjacent sections to sense probes.

Autoradiography

Autoradiograms were generated as follows. Acid-washed coverslips (no. 0, Corning, NY), previously coated with a uniform layer of photographic emulsion (NTB-2, Kodak), were apposed to the dry, hybridized sections and clamped together with a clean backing slide and binder clips under minimum sodium safelight conditions. The clamped assemblies were placed in lightproof boxes containing desiccant and exposed for 14 to 18 days at 4°C. The coverslips then were developed in Kodak Dektol developer for 2 minutes at 17°C, rinsed for 10 seconds in deionized H_2O, fixed for 3.5 minutes in Kodak Rapid-Fix, and washed in distilled water for 30 minutes. Coverslips containing autoradiograms were mounted onto microscope slides with Depex mounting

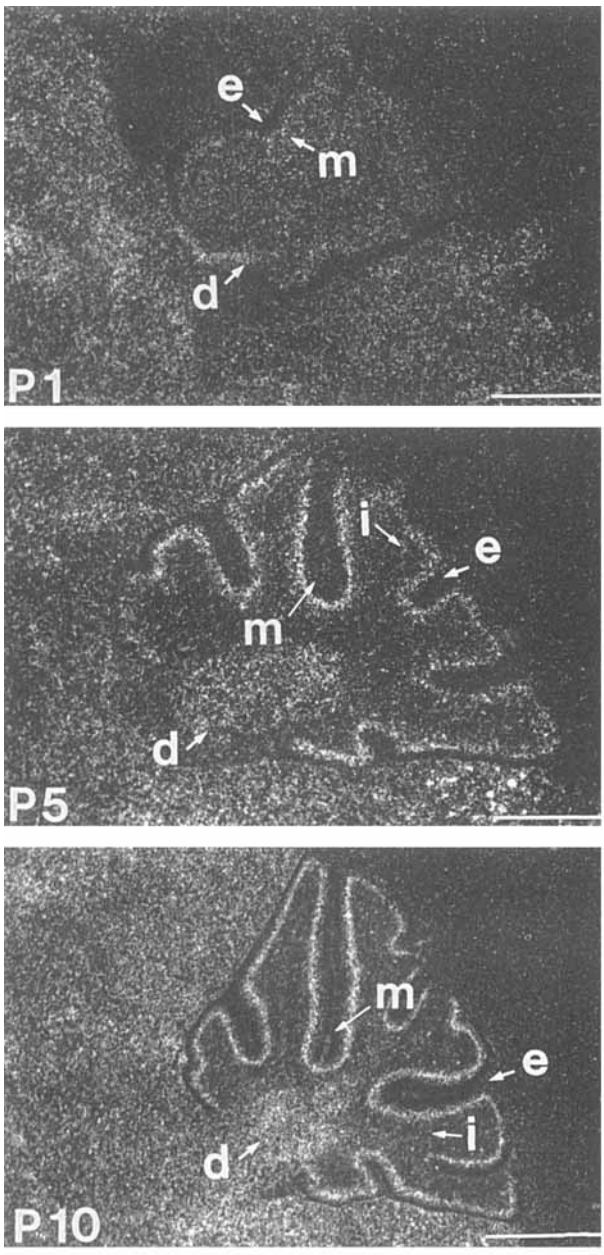

Fig. 6. The distribution of mGAT1 mRNA in the postnatal C57BL/6J mouse cerebellum. d, deep cerebellar nuclei; e, external germinal layer; i, internal granular layer; m, molecular layer. Scale bars = 500 μm in P1, P5, 1 mm in P10.

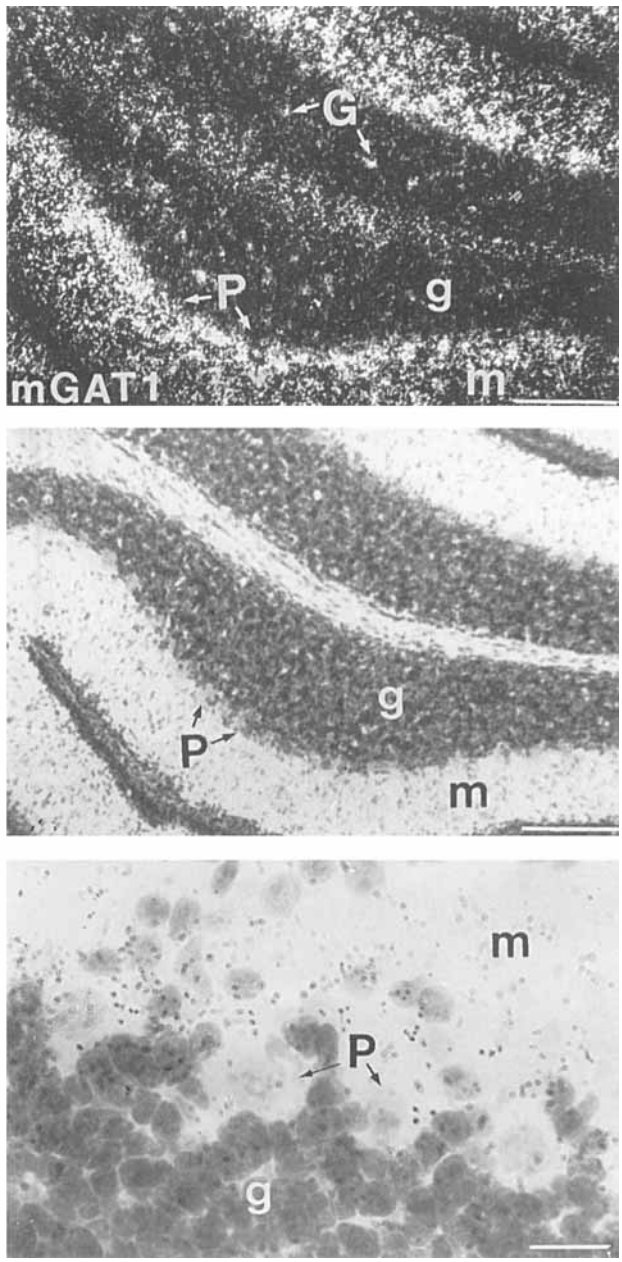

Fig. 7. **Top:** A darkfield autoradiograph showing mGAT1 expression in a single cerebellar lobule at P14. Note the high grain density at the interface of the molecular (m) and granule cell (g) layers in the vicinity of Purkinje cell bodies (P). Although granule cells are unlabeled, infrequent grain clusters scattered throughout the granule cell layer may be located over Golgi II cells (G). **Center:** The corresponding region in a nissl-stained section. **Bottom:** A region of the same section, photographed under brightfield illumination and at higher magnification, showing that Purkinje cells (P) are unlabeled. Scale bars = 200 μm in top, center, 50 μm in bottom.

medium (Bio/medical Specialties, Santa Monica, CA). In addition to the autoradiographic method described above, the slides containing the hybridized sections were dipped in the nuclear emulsion at 38°C, allowed to dry overnight, exposed for 14 to 20 days, and developed as above. Emulsion-covered sections were counterstained with cresyl violet and used for the cellular localization of the autoradiographic signal. Localizations were made with reference to Schambra et al. (1991, 1992) and Altman and Bayer (1995) in embryonic animals and with reference to Sidman et al. (1971) in adults.

RESULTS

The four murine GABA transporter mRNAs were differentially expressed throughout the CNS. The regions of highest grain density are shown in Table 1. In adult mice (Fig. 1), the mGAT1 signal was widely distributed throughout the brain and spinal cord, whereas mGAT4 mRNA was

localized in the olfactory bulb, midbrain, medulla, deep cerebellar nuclei, and spinal cord. Mouse GATs 2 and 3 were restricted almost entirely to the pia-arachnoid membrane. Because labeling at the periphery of the section could very easily be confused with an edge artifact, identically processed slides incubated with sense oligonucleotide probes were examined. Only very low, uniform grain density was observed over sense control sections, and the pia-arachnoid membrane was unlabeled. Similar results were obtained in the presence of excess unlabeled antisense probe (data not shown).

mGAT1 mRNA distribution

The expression of mGAT1 mRNA was examined in developing mice from E13 into adulthood. At E13 (Fig. 2), a low diffuse mGAT1 hybridization signal was present throughout the CNS; the ventral spinal cord and the ventricular zones immediately adjacent to the third and fourth ventricles were more densely labeled. At E14 to E18 (Fig. 3), the highest mGAT1 expression was observed in the lateral and medial regions of the ganglionic eminence and in the subventricular zone of the lateral ventricle. Grain density over the granular layer of the olfactory bulbs increased dramatically between E14 and E16 as migrating cells arrived at their adult location. Although remnants of the olfactory migratory stream remained identifiable in the subventricular zone throughout postnatal week 1, few of the migrating cells expressed the mGAT1 message by birth (Fig.3).

In postnatal animals, increasing levels of mGAT1 mRNA were distributed diffusely throughout most regions of the CNS during the first two postnatal weeks (Fig. 4). In addition, a thin band of moderate grain density, which extended over the pia-arachnoid membrane at the ventral surface of the brainstem and into the mesencephalic flexure, was retained into adulthood (Fig. 1). Clusters of high grain density were scattered throughout the hippocampus and dentate gyrus by P3, and adult levels of expression were reached by P10 (Fig. 5). By postnatal week 2, low to moderate levels of grain density were present in both cortical and subcortical regions (Fig. 4). Included in these regions were the pontine, hypothalamic, thalamic, and subthalamic nuclei as well as the basal forebrain, striatum, and substantia nigra. The hybridization signal in the granule and mitral cell layers of the olfactory bulb reached a peak by P8 (Fig. 4), after which time the grain density declined to low adult levels (Figs. 1, 4).

In the cerebellar cortex (Fig. 6), very low levels of mGAT1 mRNA were present in the Purkinje cell/molecular layer at birth; by P5, the autoradiographic grains became concentrated in a narrow band at the interface of the molecular and granule cell layers. The signal strengthened during the first two postnatal weeks as the cerebellar cortex matured (Fig. 7). When viewed at high magnification (Fig. 7, bottom panel), the majority of autoradiographic grains appeared to be associated with smaller cells, adjacent to Purkinje cell somas. Sections labeled with a ^{35}S-labeled GAD67 probe showed that glutamic acid decarboxylase (GAD), the GABA synthesizing enzyme, was present in Purkinje cells (Fig. 8). Between P10 and P14, the mGAT1 message became detectable in the molecular layer (Fig. 7), and infrequent punctate grain clusters were scattered throughout the granule cell layer in a distribution suggestive of Golgi cell labeling. Low levels of mGAT1 mRNA also were detectable in the deep cerebellar nuclei by P3, and moderate levels of expression were reached by postnatal week 2 (Fig. 6).

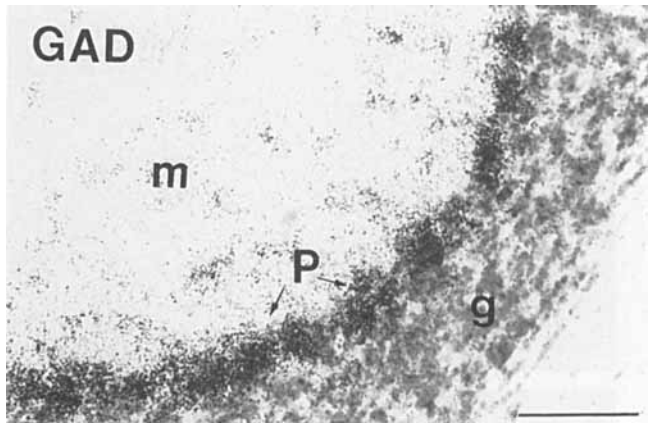

Fig. 8. Brightfield autoradiograph showing the presence of GAD67 mRNA in Purkinje cells (P) in the adult cerebellum. g, granule cell layer; m, molecular layer. Scale bar = 100 μm.

mGAT2 mRNA distribution

Although the mGAT2 hybridization signal was largely absent in the developing brain and spinal cord, at E14 a faint hybridization signal became detectable over the pia-arachnoid membrane at the ventral surface of the brainstem reaching dorsally into the mesencephalic flexure (Fig. 9). By E16, the signal was concentrated largely in the hippocampal and pontine fissures; in adults, the pia-arachnoid lining the transverse cerebral fissure was densely labeled (Fig. 9).

Although predominantly localized in the pia-arachnoid, mGAT2 mRNA also was expressed transiently in cerebellar granule cells (Fig. 10). A faint mGAT2 signal became visible in the external germinal layer at the end of the first postnatal week. The intensity of the signal increased rapidly. By P10, granule cells in the external germinal and internal granular layers were densely labeled. Expression was slightly higher in the cerebellar vermis than in the hemispheres. By P14, the external germinal layer was largely absent as granule cell proliferation and migration ceased and the mature granule cell layer was established. Although a low mGAT2 signal was retained in the adult granule cell layer, all other cerebellar lamina were unlabeled.

mGAT3 mRNA distribution

The mGAT3 hybridization signal also was localized almost entirely in the pia-arachnoid (Fig. 11) but was more intense, and more extensively distributed within the membrane, than mGAT2 mRNA. The earliest expression of the mGAT3 message was observed at E14 when the labeled membrane appeared as a filamentous network covering the surface of the brain. At E16 to birth, increasingly high grain density became visible in the transverse cerebellar, hippocampal, pontine, and olfactory fissures. Although during the third postnatal week the pia-arachnoid became considerably reduced in thickness, much of the mGAT3 signal in the pia was retained into adulthood in most brain regions. However, the mGAT3 signal in the pial membrane surround-

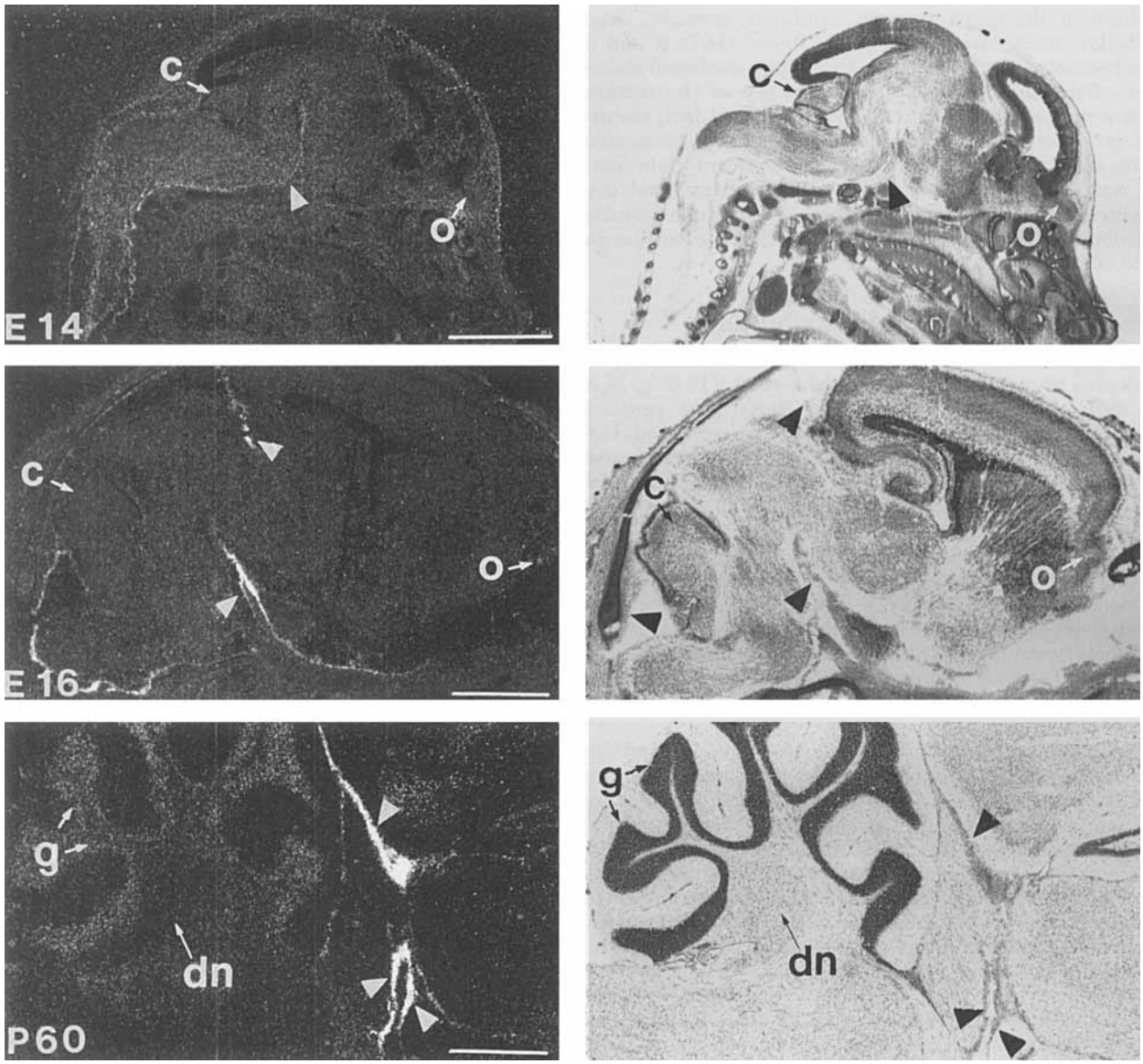

Fig. 9. Darkfield autoradiographs (left column) and corresponding cresyl violet-stained sagittal sections (right column) showing the distribution of mGAT2 mRNA in the pia-arachnoid membrane in embryonic and adult brain. In embryonic mice, mGAT2 expression was restricted to the membrane lining the pontine and hippocampal fissures and ventral brain surface (arrowheads). In adults (bottom panels), the hybridization signal became restricted to the transverse cerebral and pontine fissures (arrowheads). c, cerebellum; dn, deep cerebellar nuclei; g, granule cell layer; o, olfactory bulb. Scale bars = 2 mm in E14, 1 mm in E16, P60.

ing both the cerebellar cortex (Figs. 11, 12) and large blood vessels located near the cerebellar cortical surface (Fig. 12) decreased during postnatal week 3. By P17 (Fig. 11), only weak remnants of the hybridization signal were detectable.

mGAT4 mRNA distribution

Moderate levels of mGAT4 mRNA were distributed throughout the spinal cord and medulla by E14 (Fig. 13). The highest expression was present in the septal neuroepithelium and subventricular zone of the lateral ventricle and in the developing olfactory bulb. Grain density surrounding the ventricles increased up to E16 but was considerably reduced by E18 as cells migrated away from these regions.

During the first two postnatal weeks, mGAT4 expression increased throughout the CNS, and individual nuclei became more clearly delineated (Figs. 13, 14). The hybridization signal in the olfactory bulbs was maintained, and expression in the brainstem intensified and spread rostrally into the midbrain. Although the expression pattern was somewhat diffuse in the perinatal brain, discrete regions of high grain density became detectable over individual nuclei during postnatal week 1. These regions included the vestibular, hypoglossal, dorsal, and ventral cochlear nuclei as well as the ventral spinal cord. The facial, pontine, and inferior olivary nuclei became clearly delineated during postnatal week 2, as were the solitary nucleus and the inferior and

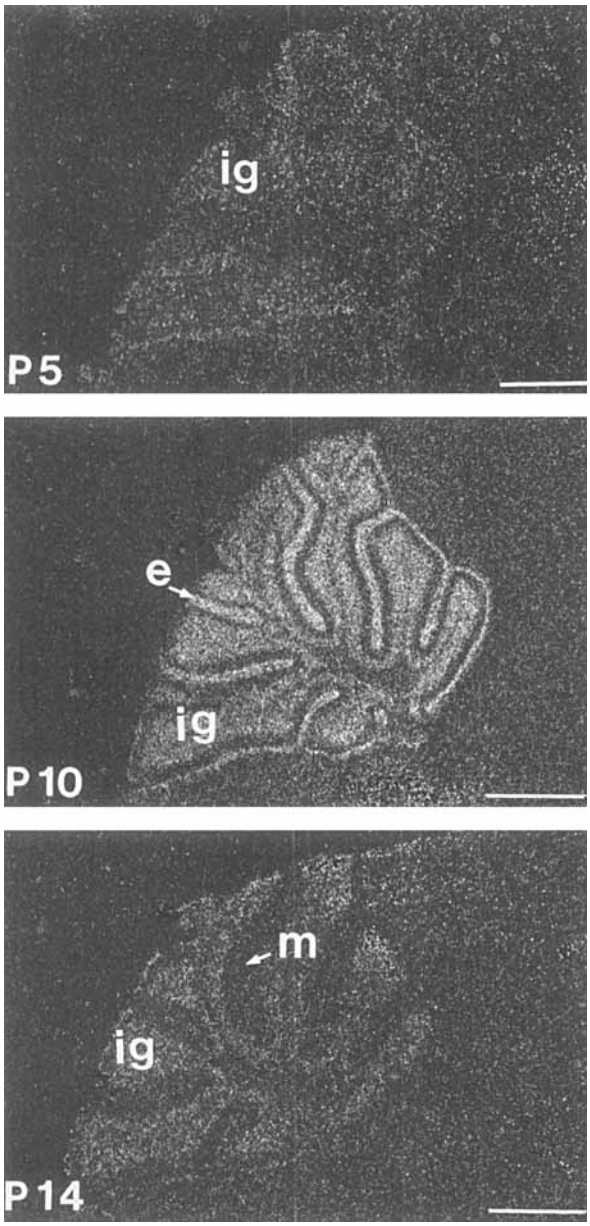

Fig. 10. Darkfield autoradiographs showing the mGAT2 signal in sagittal sections through the postnatal cerebellum. Note the sudden increase in granule cell mGAT2 labeling at P10 as granule cells in the external germinal layer (e) migrate across the molecular layer (m) to the internal granular layer (ig). Scale bars = 500 μm in P5, 1 mm in P10, P14.

superior colliculi. Labeling also increased in the cerebral cortex and in the pyramidal layer of the hippocampus and granule cell layer of the dentate gyrus (Figs. 5, 13, 14). Although the density of labeling in the brainstem continued to increase into the third postnatal week, the mGAT4 signal over the cortex and hippocampus began to decrease at the end of the second postnatal week and reached low adult levels by P20.

Within the cerebellar cortex (Fig. 15), low levels of the mGAT4 message were present over premigratory granule cells in the external germinal layer as early as P3; maximum grain density was observed between P8 and P10. The signal in the internal granular layer increased as granule cells completed their migration through the molecular layer. The deep cerebellar nuclei became densely labeled between P1 and P3; grain density increased gradually, reaching very high levels by P10. After this time, the signal decreased and only low levels were present at P60. Dense labeling in the pia-arachnoid membrane lining the transverse cerebral fissure in perinatal brains decreased by P5.

DISCUSSION

The expression patterns of four GABA transporter mRNAs were examined in late embryonic and postnatal mouse brain. Mouse GAT1 and mGAT4 mRNAs were initially expressed in ventricular and subventricular proliferative zones during embryonic week 3 and became distributed throughout the CNS during perinatal development. Although moderate levels of mGAT1 mRNA were maintained into adulthood, the mGAT4 signal in the cortex and hippocampus decreased rapidly during postnatal week 2. Nevertheless, expression in the olfactory bulbs, deep cerebellar nuclei, brainstem, and spinal cord was retained. The often diffuse nature of mGAT1 and mGAT4 expression, and low expression in the white matter, suggest that the two messages may be partially localized in glia. An additional, nonneuronal/nonglial expression pattern was observed for each of the four mRNAs. The mGAT2 and mGAT3 messages were almost entirely restricted to the pia-arachnoid membrane, and the remainder of the CNS was unlabeled with the exception of mGAT2 (which was expressed transiently in cerebellar granule cells). Although mGAT1 and mGAT4 mRNAs also were present in the pia-arachnoid, their expression was more restricted than mGAT2 and mGAT3.

Our findings in adult mice are in general agreement with earlier studies on homologous GABA transporter expression in the rat brain (Borden et al., 1992; Rattray and Priestley, 1993; Ikegaki et al., 1994; Durkin et al., 1995; Minelli et al., 1995; Itouje et al., 1996; Ribak et al., 1996), and the developmental expression patterns of GABA transporter mRNAs correspond well with the acquisition of GABA-containing cells in the developing CNS. Lauder et al. (1986) have shown that GABAergic innervation of the rat CNS occurs in a caudal to rostral direction between E12 and E16. Taking into consideration that developmental events in the mouse frequently occur slightly earlier than in the rat, the temporal and spatial expression patterns of mGATs 1 and 4 in the embryonic mouse generally follow the appearance of GABAergic fibers in the rat brain. Particularly intense GABA staining was observed in the ganglionic eminence, a proliferative zone adjacent to the lateral ventricle, in which high levels of mGAT1 and mGAT4 mRNAs were expressed during embryogenesis. During perinatal development, GABA, acting through $GABA_A$ receptors, depolarizes the neuronal membrane (Cherubini et al., 1991; Lauder, 1993; LoTurco et al., 1995), resulting in an influx of calcium (Yuste and Katz, 1991; Leinekugel et al., 1995). During the same period, neurons display a reduction in the number of primary neurites when exposed to the $GABA_A$ receptor antagonist, bicuculline (Barbin et al., 1993), indicating that GABA is required for normal neurite extension. Furthermore, Behar et al. (1994) have shown that femtomolar and nanomolar concentrations of GABA stimulate chemokinesis of embryonic neu-

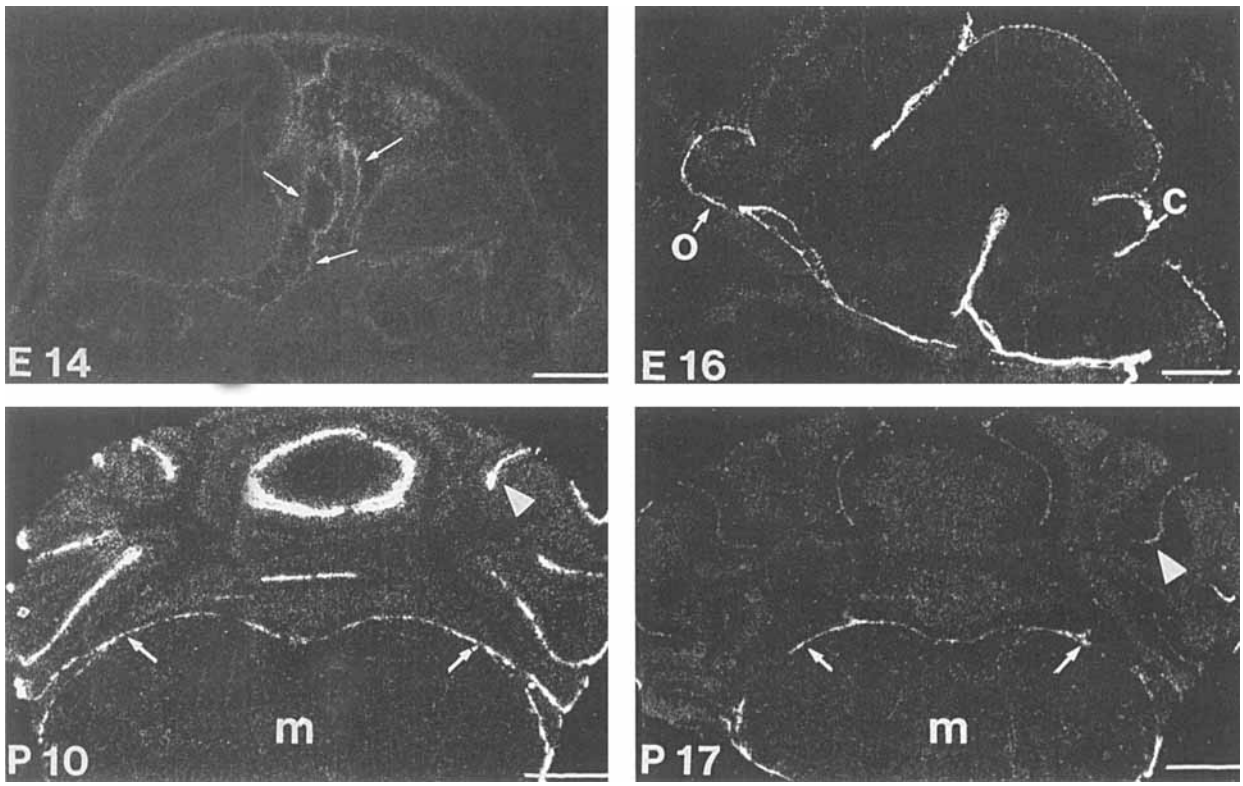

Fig. 11. Top left: A sagittal section through the E14 mouse brain showing the initial expression of mGAT3 mRNA in the pia-arachnoid membrane at the lateral brain surface (small arrows). Top right: A sagittal section at E16 showing increased mGAT3 expression in all regions of the membrane. Although the mGAT3 signal in the pia is retained into adulthood, expression in the pial membrane invaginating the cerebellar cortex (arrowheads) is lost between P10 (lower left) and P17 (lower right). c, cerebellum; o, olfactory bulb. Small arrows in bottom panels indicate labeled pial membrane at the periphery of the medulla (m). Scale bars = 2 mm in E14, 1 mm in E16, P10, P17.

rons, an action also mediated by $GABA_A$ receptors. Transporters may be crucial for releasing GABA during these early stages. Before P6, neuronal growth cones isolated from neonatal rat forebrain have been shown to extrude GABA in a calcium-independent manner. By P11, however, 50% of K+-stimulated GABA release becomes calcium dependent (Taylor and Gordon-Weeks, 1989). The calcium-independent release appears to be mediated by a reversal of the GABA transporter (Taylor and Gordon-Weeks, 1991). In recent studies, GABA was shown both to inhibit DNA synthesis and to depolarize progenitor cells in the neocortical ventricular zone (LoTurco et al., 1995). The presence of mGAT1 and mGAT4 mRNAs in embryonic proliferative zones suggests that the two transporters may have an important role in controlling regional levels of GABA in dividing and migrating cells.

Although the distributions of the mGAT transcripts generally correspond well with other GABAergic markers, there are some discrepancies. The absence of mGAT mRNA expression in cerebellar Purkinje cells was unexpected, because adult Purkinje cells contain high levels of GAD67 mRNA (Rattray and Priestly, 1993; Esclapez et al., 1994; the present study) and have GAD-immunoreactive terminals (Esclapez et al., 1994; Gilerovitch et al., 1995). Earlier studies have suggested that Purkinje cells do not accumulate [^{3}H]GABA (Storm-Mathisen, 1975) and do not, therefore, have a GABA transporter. Other studies, however, have shown that neurons resembling immature Purkinje cells take up [^{3}H]GABA as early as E13 (Hatten et al., 1984). In the present study, the strong mGAT1 signal in the vicinity of Purkinje cell bodies is localized, at least in part, in GABAergic basket cells. The basket cell terminal "pinceau" encapsulate mature Purkinje cell bodies and have been shown to contain high levels of the rat GAT1 polypeptide (Radian et al., 1990; Itouje et al., 1996). The mGAT1 hybridization signal in the perinatal molecular layer is more difficult to localize; it is unlikely to be associated with basket cells as they do not migrate into the lower molecular layer in large numbers until the end of postnatal week 1 (Fujita et al., 1966; Altman, 1975). Alternatively, GAT1 mRNA (Rattray and Priestley, 1993; Durkin et al., 1995) and its polypeptide (Radian et al., 1990) have been localized in Bergmann glia in adult rats. These cells are present in the mouse cerebellar anlage from E15 onward (del Cerro and Swarz, 1976) and are aligned with Purkinje cell bodies shortly after birth (Dupouey et al., 1985). It also is possible that, during early cerebellar development, some component of the mGAT1 signal in the cerebellar cortex is localized in Golgi cells. These cells are generated in the same ventricular region as Purkinje cells, have similar migratory pathways, and in adult mice express high levels of the mGAT1 message. The absence of mGAT expression in Purkinje cells may indicate that additional members of the GABA transporter family remain to be identified.

The developmental expression of mGAT2 and mGAT4 mRNAs in cerebellar granule cells also is anomalous. Granule cell precursors arise from neuroblasts present in the lateral caudal margin of the fourth ventricle at E13

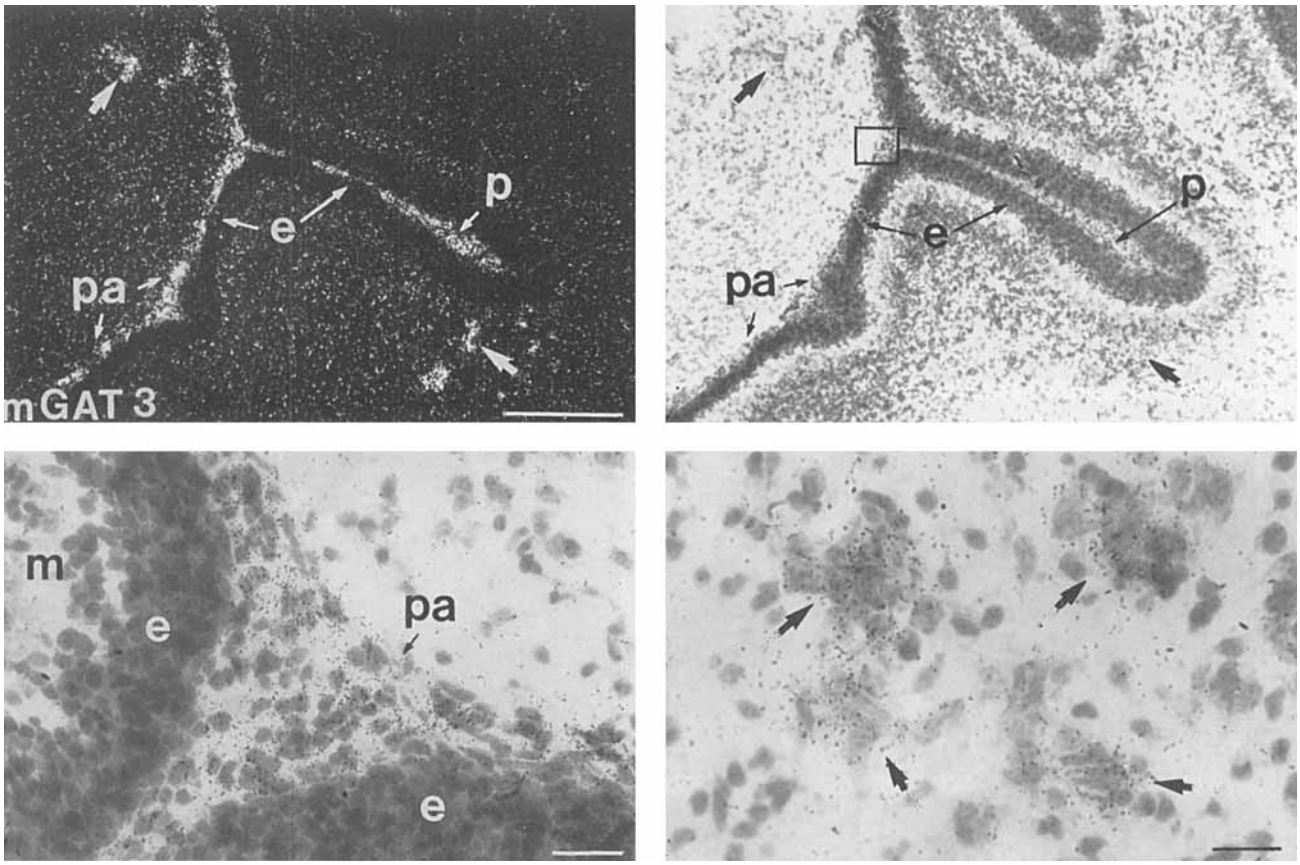

Fig. 12. Upper left: A darkfield autoradiograph showing the mGAT3 signal in the pial membrane invaginating a single cerebellar lobule at P6. Upper right: Corresponding counterstained section; labeling in the boxed region is shown at higher magnification in lower left. Upper and lower right: The cross-sectional areas of large blood vessels near the surface of the cerebellar cortex also are labeled (large arrows). e, external germinal layer; m, molecular layer; p, pial membrane; pa, pia-arachnoid. Scale bars = 200 μm in upper left, 25 μm in all others.

(Miale and Sidman, 1961); they migrate dorsolaterally and rostrally across the surface of the cerebellar plate, forming the external germinal layer of the cerebellar cortex. During postnatal week 1, the external germinal layer becomes progressively thicker, reaching its greatest width between P7 and P10 as granule cells migrate perpendicularly from the brain surface, through the molecular and Purkinje cell layers, into the internal granular layer. The granule cell uses glutamate as a neurotransmitter (Cull-Candy et al., 1988), making the function of GABA transporters in this cell type unclear. It has been suggested that homology with other transporters in the same family may allow GATs expressed in non-GABAergic cells to transport other amino acids in their vicinity (Jursky et al., 1994). Although mGATs were not expressed during the initial granule cell migration across the surface of the cerebellar anlage, mGAT2 expression in postmigratory cells in the internal granular layer occurred between P8 and P12, concurrent with both afferent and efferent synapse formation (Larramendi, 1969; Herndon et al., 1981). However, the highest mGAT4 mRNA expression occurred in granule cells well before their migration across the molecular layer, so there was little temporal correspondence between the onset of expression and synaptic contact. It is possible that neither mGAT 2 nor 4 mRNAs are translated into functional GABA transporters because in the rat cerebellar cortex, the GAT3 polypeptide (which is homologous to mouse GAT4) is absent (Ikegaki et al., 1994).

The four murine GABA transporters were expressed at varying intensities in the pia-arachnoid membrane surrounding the developing brain. The pia and arachnoid are adjacent membranes of variable width that are connected by numerous trabeculae, which traverse the fluid-filled subarachnoid space. Together, the two membranes form the leptomeninges. Whereas the arachnoid is found largely at the periphery of the brain, the pia follows the surface irregularities, lining sulci and fissures and forming sheaths around the larger blood vessels and nerves as they penetrate the brain surface (Morse and Low, 1972). The initial expression of the four transporters within the pia-arachnoid membrane at E13–14 coincides with the second of four developmental stages (McLone and Bondareff, 1975) in which vascular and meningeal elements, formed in the mesenchyme overlying the developing neuroepithelium, invest the brain surface. Although mGATs 1, 2, and 4 were present within limited regions of the membrane, mGAT3 expression was more extensive, being present both at the periphery of the brain and around large blood vessels. In most superficial brain regions, significant levels of mGAT3 expression were retained in pial cells into adulthood; however, the mGAT3 hybridization signal in the pia surrounding and invaginating the cerebellar cortex decreased during

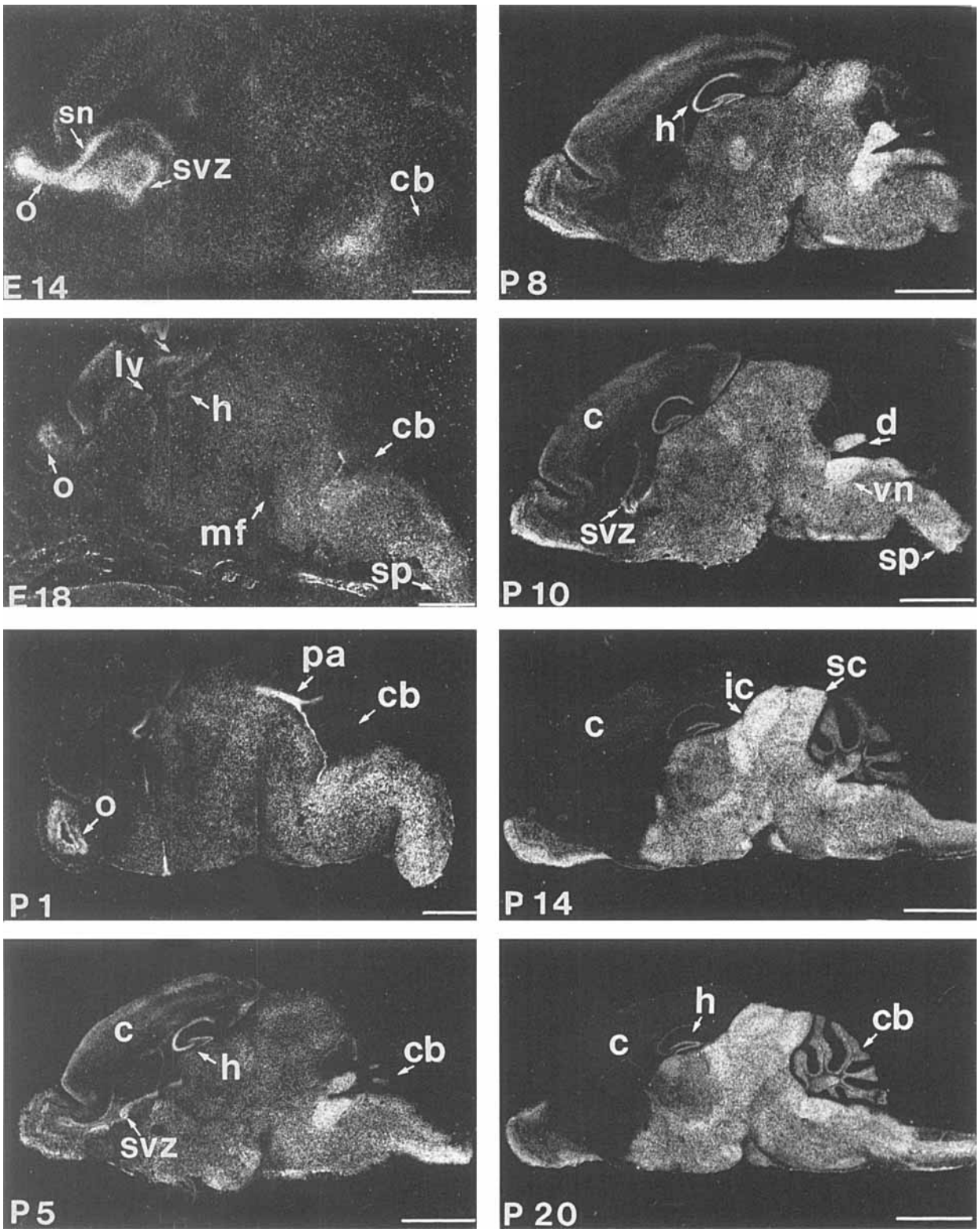

Fig. 13. Darkfield autoradiographs showing the expression patterns of mGAT4 mRNA in the embryonic and postnatal mouse brain and spinal cord. c, cerebral cortex; cb, cerebellum; d, deep cerebellar nuclei; h, hippocampus; ic, inferior colliculus; lv, lateral ventricle; mf, mesencephalic flexure; o, olfactory bulb; pa, pia-arachnoid membrane; sc, superior colliculus; sn, septal neuroepithelium; sp, spinal cord; svz, subventricular zone of the rhinencephalon; vn, vestibular nuclei. Scale bars = 1 mm in E14, E18, P1, 2 mm in all others.

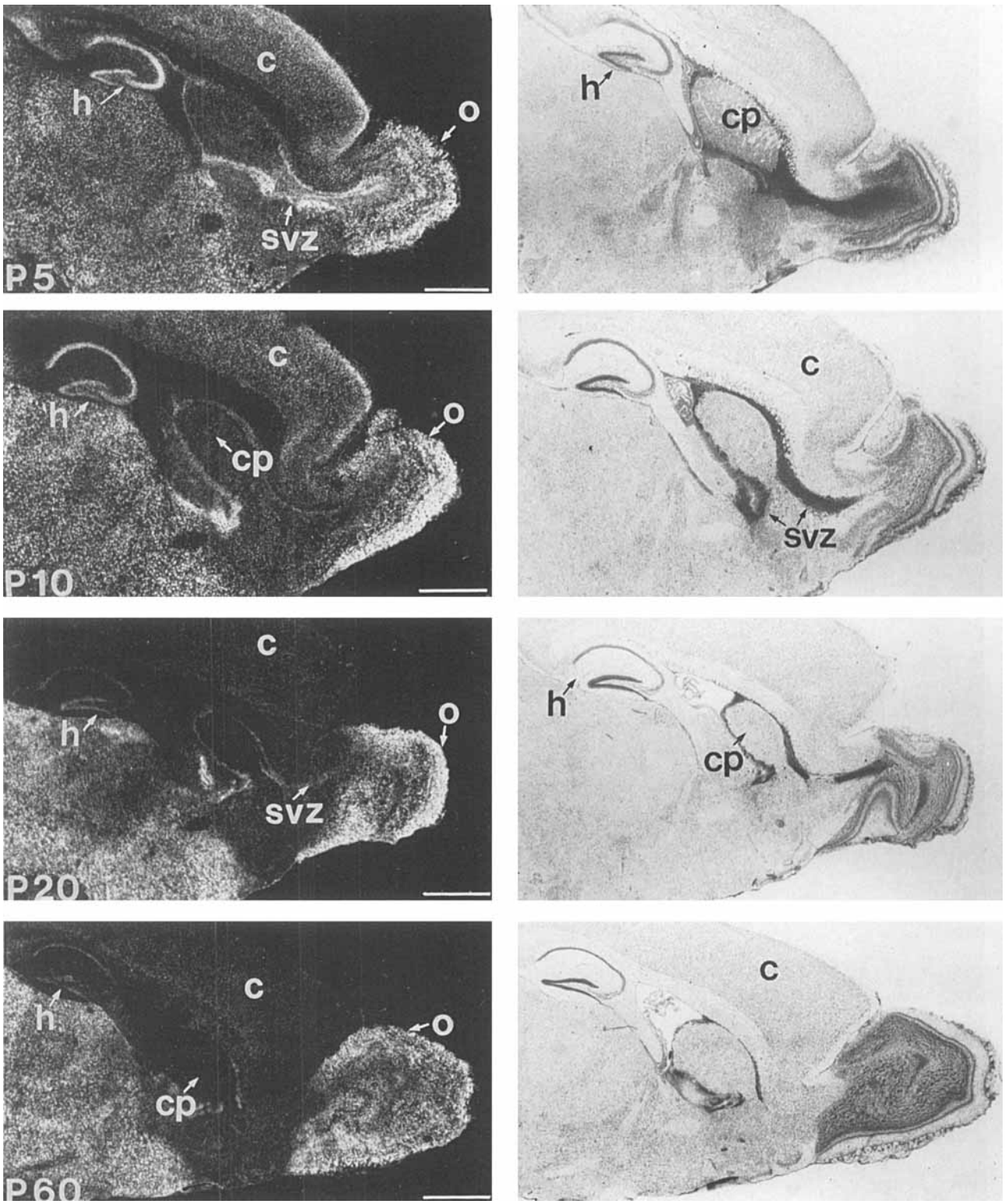

Fig. 14. Darkfield autoradiographs (left column) and corresponding cresyl violet-stained sections (right column) showing changes in mGAT4 mRNA expression in the developing forebrain from P5 to adulthood. Note the loss of the mGAT4 signal in the cerebral cortex and hippocampus during postnatal week 3. c, cortex; cp, caudate putamen; h, hippocampus; o, olfactory bulb; svz, subventricular zone of the lateral ventricle. Scale bars = 1 mm.

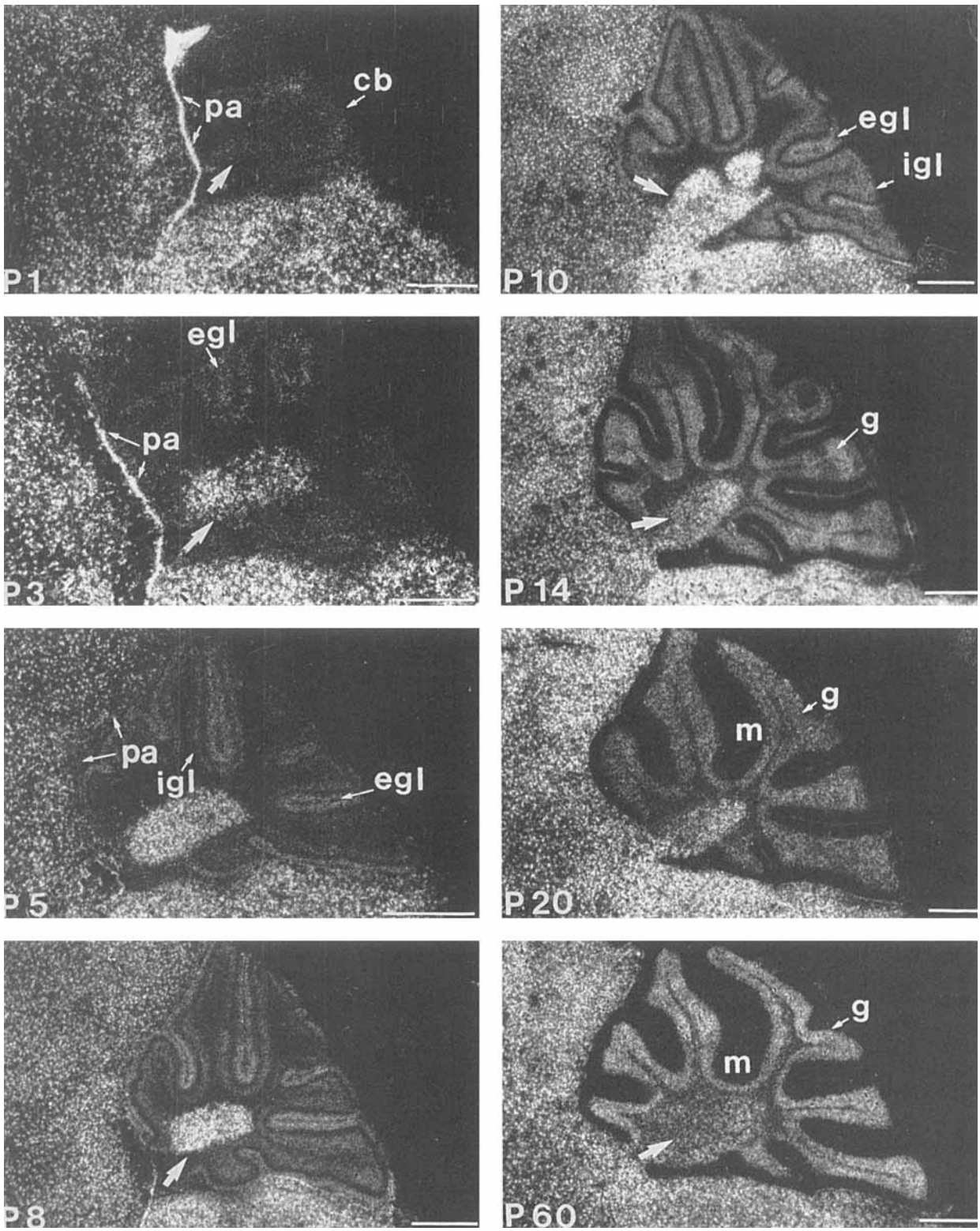

Fig. 15. Sagittal sections showing mGAT4 mRNA expression in the cerebellum during postnatal development. Note the high grain density in the pia-arachnoid membrane (pa) lining the transverse cerebellar fissure at P1 and P3. cb, cerebellum; egl, external germinal layer; g, granule cell layer; igl, internal granular layer; m, molecular layer. Large arrows indicate the deep cerebellar nuclei. Scale bars = 500 μm.

postnatal week 3 and was completely absent in adult mice. This decrease in the mGAT3 hybridization signal is likely to be due to a loss of expression in the pia, rather than the result of a general thinning of the pia-arachnoid (McLone and Bondareff, 1975). This is because the message for prostaglandin D synthase, whose polypeptide is thought to act as a sleep-regulating substance, is maintained in the cerebellar pia-arachnoid and over large blood vessels into adulthood (Urade et al., 1993). Endothelial cells surrounding blood vessels within the brain and pia have been shown to contain additional GABAergic markers, including GAD (Imai et al., 1991; Gragera et al., 1993), GABA (Lauder et al., 1986; Gragera et al., 1993) and GABA transaminase (GABA-T), the GABA metabolizing enzyme (Imai et al., 1991). It has been suggested that because GABA is unable to cross the blood-brain barrier (Krantis, 1984), it may exert a direct action on the cerebral vascular system (Gragera et al., 1993). The high levels of mGAT3 transporter in vascular system suggests a role in the removal of excess GABA from the vicinity.

Although the relationship between GABA transporters in the pia-arachnoid and developmental events within the CNS is unknown, it is possible that the high levels of mGATs in specific regions of the membrane may help to regulate the availability of GABA in superficial regions of the developing CNS. Studies using a cerebellar slice preparation (Hatten et al., 1984) have shown that GABA uptake into neurons within the E13 cerebellar anlage may only be observed after the removal of the pia-arachnoid and the choroid plexus, indicating that these structures have a high GABA-accumulating capacity. In perinatal development, there are a number of densely GABA-positive regions at the surface of the brain in close proximity to the pial membrane (Lauder et al., 1986), including the posterior commissure and adjacent regions of the tectum, and the optic recess. These areas are of particular interest as they are directly apposed to the mGAT-labeled pia-arachnoid covering the brain surface and invaginating the fissures. A variety of GABAceptive neurons, including cerebellar and olfactory granule cells and inferior olivary neurons, migrate from ventricular germinal zones over the surface of the developing brain. The majority of granule cells are completing their migration from the external germinal layer adjacent to the pial membrane into the deeper regions of the cerebellar cortex as mGAT3 expression in the cerebellar pia becomes reduced. If GABA acts as a neurotrophic and migratory agent at these developmental stages (Behar et al., 1994), the concentration of GABA close to the pia-arachnoid membrane may well be a driving force in the migration of these cells.

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