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Comparative analysis of glutamate transporter expression in rat brain using differential double in situ hybridization

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Abstract This study compares the mRNA expression pattern for the three glutamate transporters EAAC1, GLT1 and GLAST in rat brain, using a sensitive non-radioactive in situ hybridization technique. The results confirm the predominantly neuronal localization of EAAC1 mRNA, the astroglial and ependymal localization of GLAST mRNA and the astroglial and neuronal localization of GLT1 mRNA. Further, we demonstrate, using a novel differential double hybridization protocol, that the presence of GLT1 mRNA in neurons is more widespread than previously thought, and that it encompasses the majority of neurons in the neocortex, neurons in the external plexiform layer in the olfactory bulb, neurons in dorsal and ventral parts of the anterior olfactory nucleus, the majority of neurons in the anteromedial thalamic nuclei, the CA3 pyramidal neurons in the hippocampus and neurons in the inferior olive. In addition, we demonstrate marked variations in the expression levels of GLT1 and GLAST mRNAs in different brain areas, suggesting that their mRNA levels are regulated by different mechanisms. Finally, for EAAC1 we demonstrate also a widespread distribution and a marked heterogeneity in the expression levels. EAAC1 is strongly expressed by a heretofore unrecognized group of cells in white matter tracts such as the corpus callosum, fimbria-fornix or anterior commissure. Also, strong EAAC1 expression is present in groups of scattered cells in grey matter areas of much of the forebrain and the cerebellum. These results provide more detailed information about the precise cellular localization of these three glutamate transporters and their regulation at the mRNA level.

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Introduction

High-affinity glutamate transporters play an important role in the central nervous system. They terminate the excitatory action of synaptically released glutamate through reuptake and maintain extracellular glutamate below neurotoxic levels. Furthermore, glutamate transporters are responsible for shuttling glutamate into cells that need this amino acid as precursor, e.g., for synthesis of the neurotransmitter GABA in GABAergic neurons. Several glutamate transporters have been isolated and characterized over the last five years, namely EAAC1 (Kanai and Hediger 1992), GLT1 (Pines et al. 1992), GLAST (Storck et al. 1992), EAAT4 (Fairman et al. 1995) and EAAT5 (Arriza et al. 1997). Of these, GLT1, GLAST and EAAC1 have a wide distribution in the CNS, while EAAT4 and EAAT5 are mostly restricted to the cerebellum and the retina, respectively.

The cellular expression of EAAC1, GLT1 and GLAST mRNA has been investigated in a number of studies, with ever more refined results. Early studies using radioactive in situ hybridization established that EAAC1 is expressed exclusively by neurons, while GLT1 and GLAST are predominantly expressed by astrocytes (Storck et al. 1992; Torp et al. 1994; Kanai et al. 1995; Kiryu et al. 1995; Sutherland et al. 1996; Velaz-Faircloth et al. 1996). More recent studies, which used non-radioactive in situ hybridization, have found that GLAST is also expressed by ependymal cells and that GLT1 mRNA is also found in some neuronal subgroups, including CA3 pyramidal neurons in the hippocampus, neurons of layer VI and the endopiriform nucleus in the cerebral cortex, and, possibly, a subset of neurons in the thalamus (Schmitt et al. 1996, 1997; Torp et al. 1997). The demonstration of GLT1 mRNA in neurons raises the important issue of how the expression of these transporters is regulated and of the physiological significance of

this differential expression. While the neurons in CA3 of the hippocampus clearly express both EAAC1 and GLT1, it is unclear whether the other neuronal subgroups that express GLT1 also express EAAC1. Furthermore, it is not clear to what extent the expression of GLT1 and GLAST message overlaps in astrocytes.

We have recently employed an improved protocol for non-radioactive in situ hybridization in the study of glutamate transporter expression in the CNS. This protocol is based on the method of Schaeren-Wiemers and Gerfin-Moser (1993) and provides superior sensitivity and cellular morphology compared to previously employed methods. In addition, we have incorporated the novel tyramide-signal amplification technique (Adams 1992) into our protocol, which allows the visualization of in situ hybridization signals with fluorescent markers. Thus, using these new methods we are able to perform sensitive double-in situ localization of two different transporter mRNAs in the same tissue section. We have used these techniques to re-evaluate the distribution of EAAC1, GLT1 and GLAST expression in rat brain. In particular, we have mapped the neuronal subpopulations that express GLT1 message, and we have determined whether all GLT1-expressing astrocytes also express GLAST and whether GLT1-expressing neurons also express EAAC1 message.

Materials and methods

Male Sprague-Dawley rats (200–300 g, Taconic Farms) were used, which were kept in a temperature- and light-controlled room (7 am–5 pm light) with free access to rat chow and water before sacrifice. Rats were terminally anesthetized with pentobarbital and then sacrificed by decapitation, using procedures approved by the Harvard Medical School Animal Welfare Committee. Brains were dissected and snap-frozen in isopentane at –30°C and then either processed immediately or stored at –80°C. All chemicals, unless otherwise noted, were obtained from Sigma (St. Louis, Mo.).

Generation of cRNA probes

The cRNA probes for in situ hybridization analysis were prepared from expression plasmids (pBluescript II SK^-) containing the transporter sequences. The EAAC1 probe consisted of nucleotides 500–1751 of the EAAC1 sequence (Kanai et al. 1995), the GLT1 probe contained the whole transcribed GLT1 sequence (Pines et al. 1992), and the GLAST sequence contained nucleotides 58 to 2259 (Storck et al. 1992). The EAAC1 and GLT1 probes were transcribed from linearized plasmids using the Boehringer-Mannheim Genius kit (Indianapolis, Ind.). To generate the GLAST probe, a cDNA fragment was first synthesized from the plasmid containing the GLAST sequence, using PCR and two primers that consisted of RNA polymerase initiation site sequences (20 nucleotides) linked to the GLAST specific sequence (25 nucleotides). Subsequently, the GLAST probes were directly transcribed from the cDNA fragment. The EAAC1 and GLAST probes were labeled with digoxigenin (DIG)-UTP, while the GLT1 probes were labeled with either DIG-UTP or fluorescein (FITC)-UTP. After transcription all probes were alkali-hydrolyzed to an average length of 250 base pairs (Schaeren-Wiemers and Gerfin-Moser 1993).

In situ hybridization

The in situ hybridization procedure was based on the method of Schaeren-Wiemers and Gerfin-Moser and includes several modifications. Coronal and sagittal sections of brain (10–12 μm) were cut on a cryostat and captured onto positively charged microscope slides (Superfrost Plus, Fisher Scientific). The slides were either processed immediately or stored at –80°C. To process the slides, they were adjusted to room temperature, and then fixed in freshly depolymerized, ice-cold 4% paraformaldehyde in 0.1 M PBS for 15 min, rinsed 3 times for 5 min in PBS, acetylated in 0.1 M triethanolamine/acetic anhydride for 10 min, rinsed twice in PBS and once in 2×SSC for 5 min each, before they were submerged in slide mailers in hybridization buffer [50% formamide (Life Technologies, Gaithersburg, Md.), 5×SSC, 2% blocking reagent (Boehringer), 0.1% SDS, and 0.02% N-laurylsarcosine] for 1–3 h. The cRNA probes were boiled in 20 μl of hybridization buffer for 2 min before they were added to the slide mailers. Probe concentrations approximated 100 ng/ml for EAAC1 and GLAST, and 30 ng/ml for GLT1. Hybridization proceeded in the mailers overnight in a water-bath adjusted to 70°C. Slides were removed from the mailers and placed in coplin jars in 2×SSC for three quick rinses, and then in 0.2×SSC for two 30 min rinses, all at 70°C. Subsequently, sections were blocked in 0.1 M maleate buffer (pH 7.0) containing 1% blocking reagent for 1 h at RT, and then incubated in sheep anti-DIG-Fab fragments conjugated to alkaline phosphatase (0.15 U/ml, Boehringer) in maleate/blocking buffer for 1 h. After several rinses over 1 h in maleate, and one quick rinse in 0.1 M TRIS, 0.15 M NaCl, pH 9.5, slides were placed upside down onto a tray and developed in the dark in substrate solution (pH 9.5) containing 0.1 M TRIS, 0.15 M NaCl, 50 mM $MgCl_2$, 1 mM levamisole, 0.4 mM 5-bromo-4 chloro-indolylphosphate (BCIP) and 0.4 mM nitroblue tetrazolium chloride (NBT, both from Boehringer). Development times ranged from 4 h for GLT1 to 18 h for GLAST and EAAC1. The development was stopped by incubation in 10 mM TRIS, 25 mM EDTA pH 8.0. Slides were quickly rinsed in water and then coverslipped with Vectashield (Vector).

For double in situ hybridization labeling, DIG-labeled EAAC1 or GLAST probe was combined with FITC-labeled GLT1 probe during the hybridization. After the blocking step in maleate/blocking buffer, sections were first treated with the avidin/biotin blocking kit (Vector, Burlingame, Calif.) and then incubated for 1 h in a mix of anti-DIG-alkaline phosphatase conjugate (0.15 U/ml) and mouse anti-FITC antibodies (0.1 μg/ml, Boehringer). Subsequently sections were rinsed in maleate, and then incubated for 1 h in biotinylated anti-mouse secondary antibodies (1:500, rat adsorbed, Jackson Immunoresearch), which was followed by rinses first in maleate, and then TNT (0.1 M TRIS, 0.15 M NaCl, 0.05% Tween-20), and a 30 min incubation in avidin-horseradish peroxidase conjugate (1:500, NEN tyramide amplification kit, Boston, Mass.) in TRIS-NaCl buffer containing 0.5% blocking reagent (NEN kit). Following rinses in TNT, the signal was amplified using biotin-tyramide for 10 min in amplification buffer (NEN kit). Subsequently, sections were rinsed in TNT and TRIS-NaCl (pH 9.5), and then developed in NBT/BCIP (see above). Following incubation in TRIS-EDTA, slides were incubated in streptavidin-CY3 (1:200, Zymed) for 1 h, rinsed in TRIS-NaCl and water, and then coverslipped with Vectashield (Vector).

As controls, sections were hybridized with sense cRNA probes or without any probe. To control for crossreactivity between the probes for EAAC1 and GLT1, and between those for GLAST and GLT1, sections were hybridized either with a mixture of EAAC1 or GLAST antisense RNA and an excess of GLT1 sense RNA, or with a mixture of GLT1 antisense RNA and an excess of EAAC1 or GLAST sense RNA.

Results

Single-labeling procedure

The probes for all three transporter proteins yielded specific signals that are in agreement with previous findings. The use of sense cRNA as a probe yielded no signal for

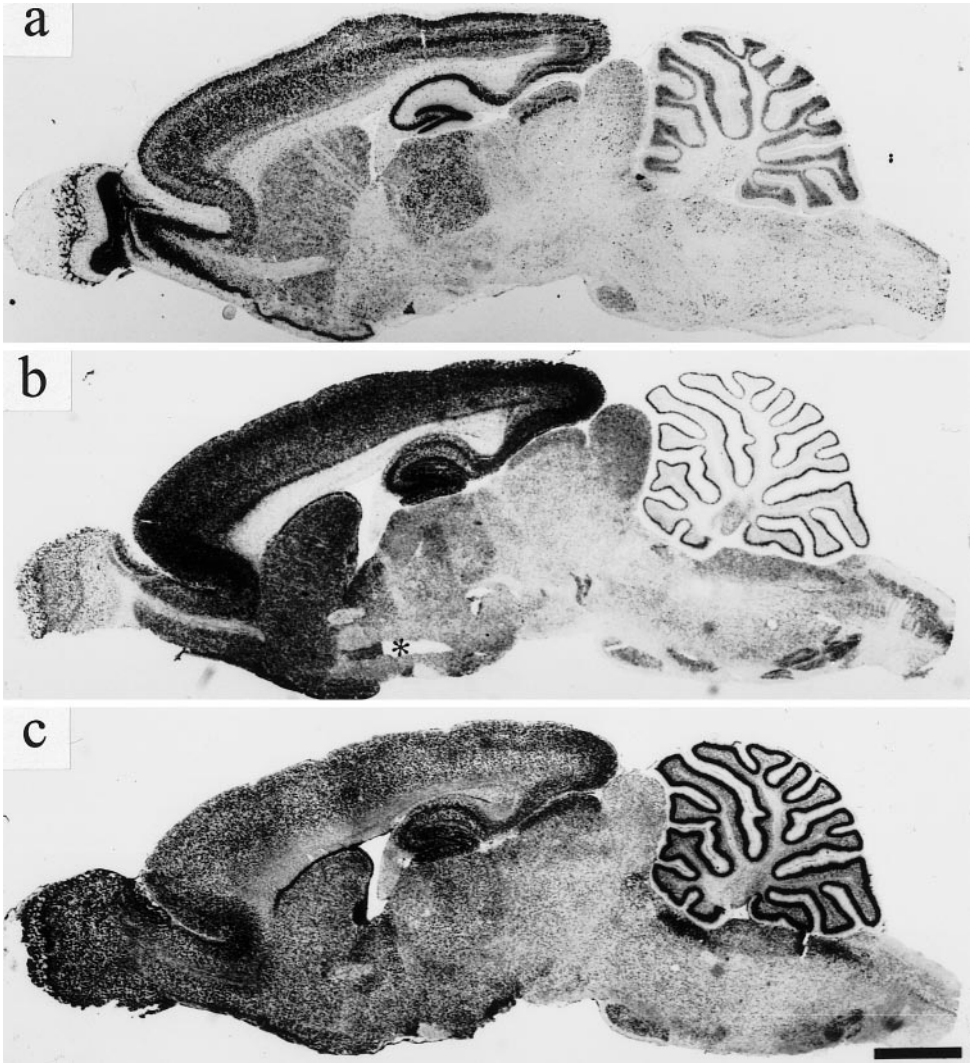

Fig. 1 Photomicrographs of sagittal sections of rat brain hybridized with the EAAC1 (**a**), GLT1 (**b**), and GLAST (**c**) cRNA probes. The development time for the GLT1 labeling was 4 h, while that for EAAC1 and GLAST labeling was 18 h. Note the relatively higher labeling intensities with all probes in the cerebral cortex, hippocampus and ventral forebrain than in the midbrain and brainstem. Also, GLT1 labeling is relatively low in the olfactory bulb, while that of GLAST is of the same intensity in the olfactory bulb as in the cortex (* in **b**, denotes tear in section). *Bar* 2.5 mm

any of the three transporters. In addition, we tested potential crossreactivities between EAAC1 and GLT1, and between GLAST and GLT1, by incubating the EAAC1 or GLAST antisense probes with an excess of GLT1 sense probe, and by incubating GLT1 antisense probes with an excess of EAAC1 or GLAST sense probe. An excess of GLT1 sense probe did not affect the EAAC1 or the GLAST labeling, while an excess of EAAC1 or GLAST sense probe did not affect the GLT1 labeling, thus ruling out the possibility of cross-hybridization.

The incubation in slide mailers resulted in intense homogenous signals with virtually no background. This is in contrast to the procedure of covering the slides with parafilm during hybridization, which causes background labeling, e.g., for GLT1 in CA1 pyramidal cells in the hippocampus (Torp et al. 1997; U. V. Berger, unpublished observation). Complete submersion in slide mailers may be equivalent to covering the slide with hybridization solution in a humidified chamber, but it has the advantage of eliminating the possibility that the hybridization solution evaporates and that its composition changes. In addition, our method provides higher sensitivity and improved cellular morphology compared to previous methods. For example, labeling of astrocytes or neurons could easily be distinguished; astrocytes displayed the characteristic labeling of their processes, while neuronal labeling was restricted to the cell bodies. In previous studies using nonradioactive or radioactive methods of hybridization, this difference was not as easily observed (Torp et al. 1994; Velaz-Faircloth et al. 1996; Torp et al. 1997). This significant improvement results from not dehydrating the sections through graded alcohol series (which reduces the sensitivity by about one order of magnitude, see Schaeren-Wiemers and Gerfin-Moser 1993), and from using long cRNA probes that have been hydrolyzed to an average length of 250 base pairs.

The substrate development for EAAC1 and GLAST was allowed to process for 18 h, while that for GLT1, because of its high abundance, was stopped at 4 h. Figure 1 shows mRNA labeling for EAAC1 (a), GLT1 (b), and GLAST (c) in sagittal sections of rat brain. All three transporter messages are expressed at high levels in the cerebral cortex, hippocampus, striatum, the ventral forebrain and thalamus. More caudal regions such as the

midbrain, pons, and medulla oblongata show generally lower labeling intensities. Cell groups showing high levels of EAAC1 labeling include layer V of cerebral cortex, the pyramidal and granular layers of the hippocampus, the granular layer in the olfactory bulb, the pyramidal cells of the piriform cortex, the supraoptic nucleus, and the deep layer in the superior colliculus. GLAST mRNA is expressed at very high levels in Bergmann glia of the cerebellum, as previously reported (The difference in signal intensity between the cerebellum and the rest of the brain does not appear as pronounced as in previous studies, possibly due to saturation of the labeling signal in the Bergmann glial cells). The distribution of GLT1 and GLAST mRNA is generally similar, yet there are some marked differences. For example, the message levels in the olfactory bulb are relatively low for GLT1 and high for GLAST (as compared to the cerebral cortex), while in the inferior colliculus, labeling is strong for GLT1 but weak for GLAST (as compared to surrounding regions). Further, GLAST mRNA is strongly expressed in the trigeminal and vestibular nuclei, whereas GLT1 is strongly expressed in the inferior olive (as compared to other regions of the brainstem).

Double-labeling procedure

The tyramide signal amplification created intense and specific signals for GLT1 that were easily observed with a regular fluorescence microscope. No background was observed when the cRNA probes were omitted. Also, the labeling patterns seen with fluorescent detection were identical to those observed with conventional detection at the brightfield level. We decided to use the indirect biotin-tyramide amplification with subsequent streptavidin-CY3 incubation rather than the direct method which uses FITC-labeled tyramide. The indirect method resulted in more intense signals, and decreased the chance that the fluorescent signal may be quenched during the alkaline phosphatase reaction. In previous double-in situ procedures, where both signals were observed under brightfield illumination, the exact determination of colocalization was often difficult because of mixing of the signals, or the labeling had to be performed sequentially. Our new method allows the precise determination of two different mRNAs in the same cell without the problem of signal mixing. Naturally, if a cell contains large amounts of the mRNA detected by alkaline phosphatase/NBT/BCIP, then the accumulation of BCIP/NBT precipitate may prevent access of streptavidin-CY3 to the biotin-tyramide-labeled second mRNA, and thus may block the detection of the second mRNA in the same cell. This was not considered a drawback, since it can be easily prevented by shortening the development time of the alkaline phosphatase reaction. Rather, it was actually an advantage that allowed the differential demonstration of GLT1 in either neurons or astrocytes. When the GLT1 probe was used in combination with the GLAST probe, strong GLAST signals blocked the GLT1 labeling in astrocytes and led to a clear depiction of only the GLT1 mRNA expressed in neurons. Similarly, when GLT1 and EAAC1 probes were combined, strong EAAC1 labeling blocked the GLT1 signals in neurons without affecting the astrocytic GLT1 labeling. By comparing the results obtained with the combination of GLT1 and EAAC1 probes with the combination of GLT1 and GLAST probes, we were able to clearly identify the neuronal cell populations that express GLT1. A schematic view of this differential double-in situ procedure is shown in Fig. 2.

Regional distribution of EAAC1, GLT1 and GLAST

Results were obtained from sets of three adjacent or closely spaced sections (cut either coronally or sagittally), that were hybridized with a mixture of EAAC1-DIG and GLT1-FITC, a mixture of GLAST-DIG and GLT1-FITC, or with GLT1-DIG alone. The DIG-labeled probes were visualized at the light microscopic level with the alkaline phosphatase substrate NBT/BCIP, and the FITC-labeled probe was visualized at the fluorescent level with CY3.

Neocortex

Figure 3A–E shows the distribution of EAAC1, GLT1 and GLAST mRNA in a sagittal 10 μm section through frontal isocortex 1 and 2 (Zilles and Wree 1995) at approximately 1.4 mm lateral and –1 mm to Bregma (Plate 80, Paxinos and Watson 1986). In order to facilitate the direct comparison, the pictures A–E were arranged in the following order: EAAC1-DIG (Fig. 3A, showing neurons); GLT1-FITC, co-hybridized with GLAST-DIG (Fig. 3B, showing mostly neurons); GLT1-DIG (Fig. 3C, showing astrocytes and neurons); GLT1-FITC, co-hybridized with EAAC1-DIG (Fig. 3D, showing mostly astrocytes); GLAST-DIG (Fig. 3E, showing astrocytes). EAAC1 is expressed by neurons throughout layers II to VI. In agreement with previous studies (Kanai et al. 1995; Velaz-Faircloth et al. 1996; Torp et al. 1997), neurons in layers II and V are more strongly labeled than those in the other layers (Fig. 3A). In addition, strong labeling was found in a previously unidentified group of neurons which was scattered throughout the cortex and the underlying corpus callosum (see below). GLAST mRNA is strongly and relatively homogeneously expressed by astrocytes throughout all layers of the cortex (Fig. 3E). The distribution of GLT1 mRNA as detected by the DIG-labeled probe and NBT/BCIP development is shown in Fig. 3C. The expression of GLT1 appears both in astrocytes and neurons, but the extent of neuronal GLT1 is difficult to assess. The expression of GLT1 in neurons is much more easily recognized in a section co-hybridized to GLAST (Fig. 3B), in which the astrocytic GLT1 labeling is obscured by the GLAST signal. Comparison with the EAAC1-labeled section shows that high levels of GLT1 mRNA are contained in neurons in layer VI, and somewhat lower levels are present in neurons in layers IV

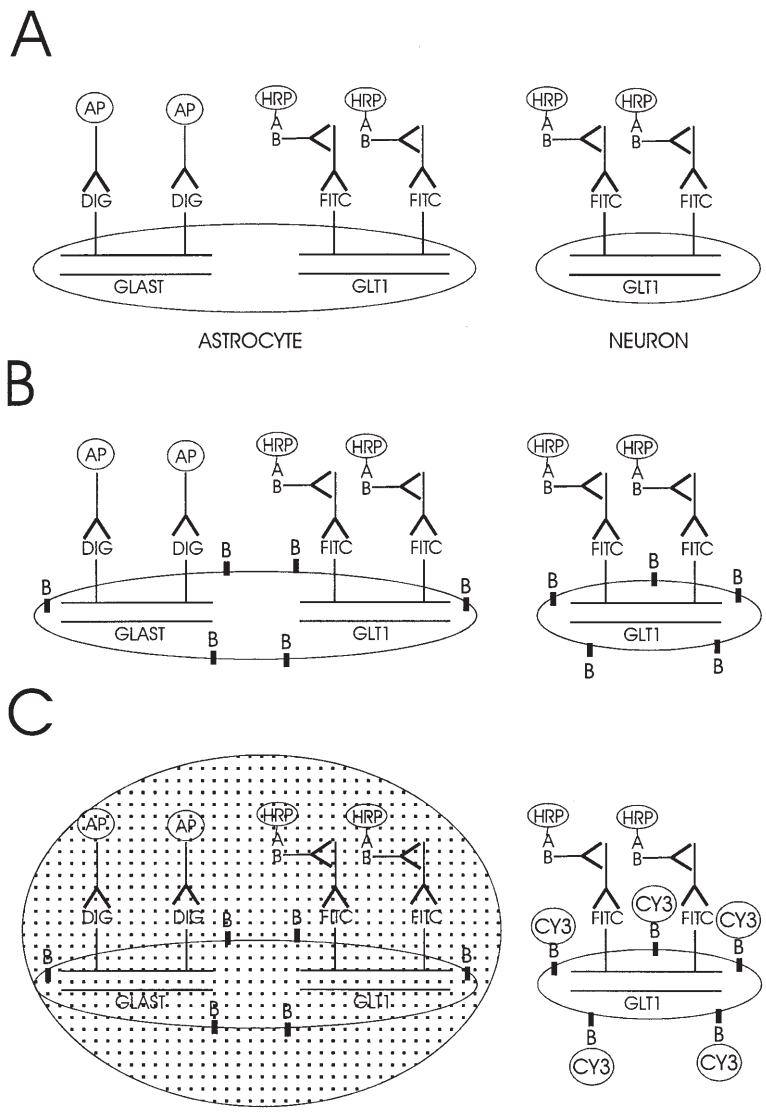

Fig. 2A–C Schematic view of the differential double-in situ procedure using GLT1 and GLAST probes. **A** GLAST mRNA in astrocytes is hybridized to DIG-labeled GLAST antisense probe, which is then detected using anti-DIG-Fab fragments conjugated to alkaline phosphatase. GLT1 mRNA in astrocytes and neurons is hybridized to FITC-labeled GLT1 antisense probe, which is then detected sequentially with anti-FITC antibodies, followed by biotinylated anti-mouse antibodies, and avidin-horseradish peroxidase. **B** The tyramide signal amplification with biotinyl-tyramide as substrate is performed. In this step, horseradish peroxidase catalyzes the deposition of biotin-tyramide (**B**) in GLT1-positive cells. **C** The alkaline phosphatase reaction is performed, which precipitates the NBT/BCIP substrate in GLAST-positive cells. The reaction is allowed to proceed until a strong signal is obtained that covers the whole cell (indicated by *shading*). Subsequently, streptavidin-CY3 is added that labels only the biotin-tyramide in neurons. The access of streptavidin-CY3 to the biotin-tyramide in astrocytes is blocked by the NBT/BCIP precipitate

and V. Very low levels are present in neurons of layers II and III. Previous studies using non-radioactive in situ hybridization have also described GLT1 mRNA expression in neurons in layer VI (Schmitt et al. 1996; Torp et al. 1997) or in layer II of the medial entorhinal cortex (Torp et al. 1997). Using our sensitive double-labeling method, we can now demonstrate that the neuronal GLT1 expression in the cortex is more widespread than previously thought. Figure 3D shows the fluorescent GLT1 labeling in a section co-hybridized with EAAC1. Here part of the weaker neuronal labelling of GLT1 in the upper layers is obscured by the EAAC1 signal; however, the astrocytic GLT1 labeling is visible undisturbed.

The neuronal expression of GLT1 was observed throughout all regions of the neocortex from the frontal pole to the subiculum leading into the hippocampus. Particularly strong labeling was found in the dorsal endopiriform nucleus, in agreement with a previous study (Schmitt et al. 1996). The observation that co-hybridization with GLAST prevented astrocytic labeling of GLT1 in grey matter of the neocortex indicates that all astrocytes that express GLT1 in this region also express GLAST. In the same vein, the blocking of the neuronal GLT1 labeling as a result of co-hybridization with the EAAC1 probe suggests that the affected neurons express both EAAC1 and GLT1 message. In fact, examination of the double-labeled sections under high magnification suggests that the majority of neurons that express GLT1 also express at least some amount of EAAC1. Figure 4A, C, E shows cells in the deep part of layer VI of cortex double-labeled for EAAC1 and GLT1. Figure 4A shows the EAAC1 brightfield signal and Fig. 4E the GLT1 fluorescent signal. Figure 4C shows the two signals simultaneously, demonstrating that most neurons that express GLT1 message also express EAAC1 message. Since GLAST mRNA levels are lower in white matter than in grey matter, the blocking of GLT1 signals was not as complete in these regions. However, closer examination of these areas suggested that all GLAST-positive astrocytes were also labeled for GLT1.

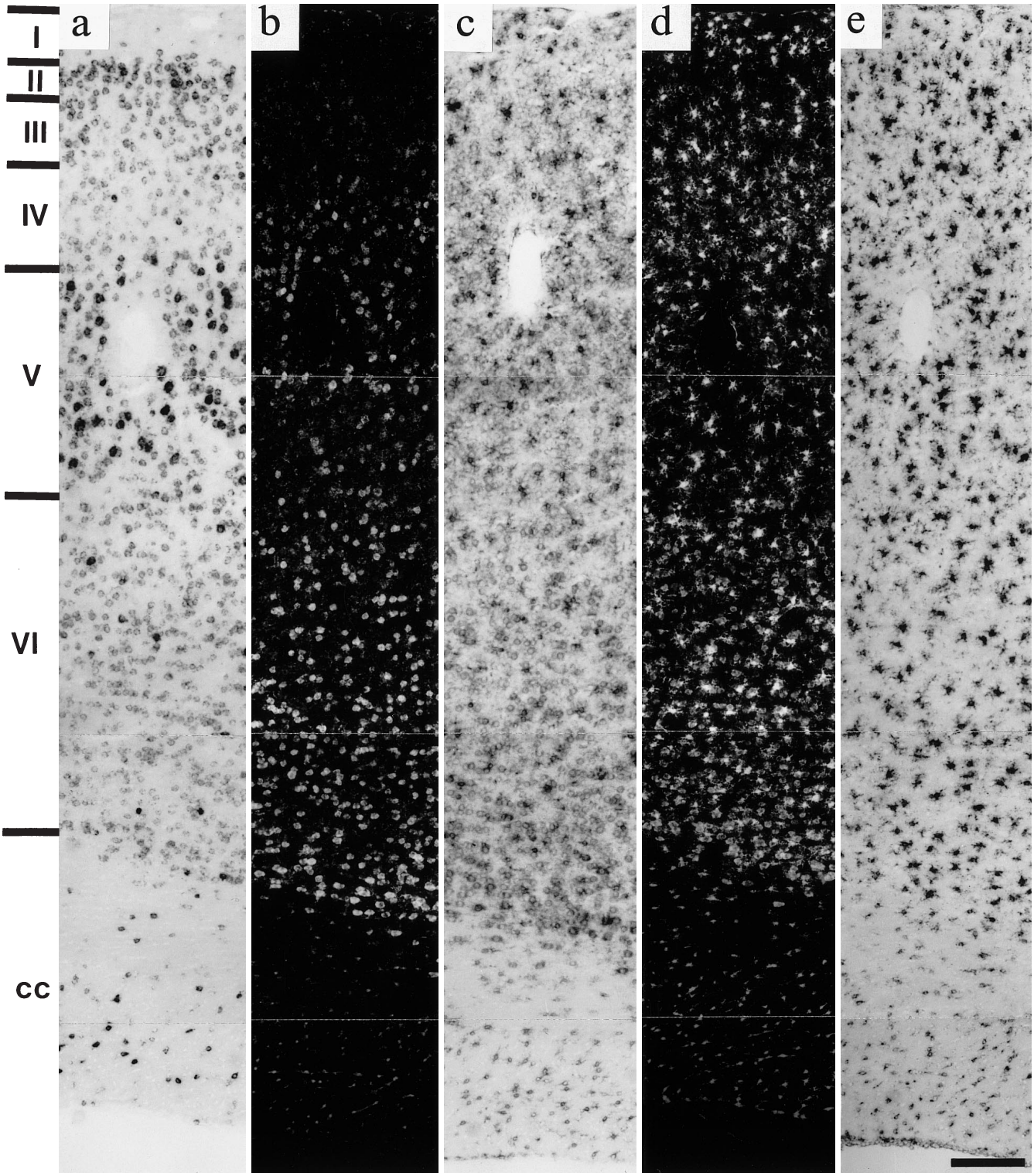

Fig. 3a–e Distribution of EAAC1, GLT1 and GLAST mRNA in cerebral cortex, as detected by differential double-in situ hybridization. Sections were hybridized with either a mixture of DIG-labeled EAAC1 and FITC-labeled GLT1 probes (**a, d**), a mixture of DIG-GLAST and FITC-GLT1 probes (**e, b**), or with DIG-GLT1 probe alone (**c**). **a, c, e**, Show alkaline phosphatase labeling of DIG-labeled probes; **b, d** show fluorescent labeling of FITC-labeled GLT1. **a** EAAC1 labeling is present in neurons throughout layers II to VI of the cortex and in cells in the corpus callosum. Neurons in layer V (pyramidal cells) and layer II show the highest labeling intensities. Note the scattered cells in the cortex and corpus callosum that are strongly expressing EAAC1. **b** Neuronal GLT1 labeling in a section co-hybridized with GLAST probe; the astrocytic GLT1 labeling is obscured by the alkaline phosphatase product of the GLAST signal (see **e**). Note that neuronal GLT1 labeling is strongest in the deep part of layer VI, moderate in layers IV and V, and very weak in layers II and III. **c** Single GLT1 labeling. The signals are present in astrocytes and neurons, yet the extent of neuronal labeling is difficult to assess. **d** GLT1 labeling in section co-hybridized with EAAC1 probe, the signals are strongest in astrocytes, but neuronal labeling predominantly in layer VI can also be distinguished. **e** GLAST labeling is present in astrocytes at a high intensity throughout all layers of the cortex. Note that the labeling intensity for both GLT1 and GLAST is lower in the corpus callosum than in the cortex. *Bar* 200 μm

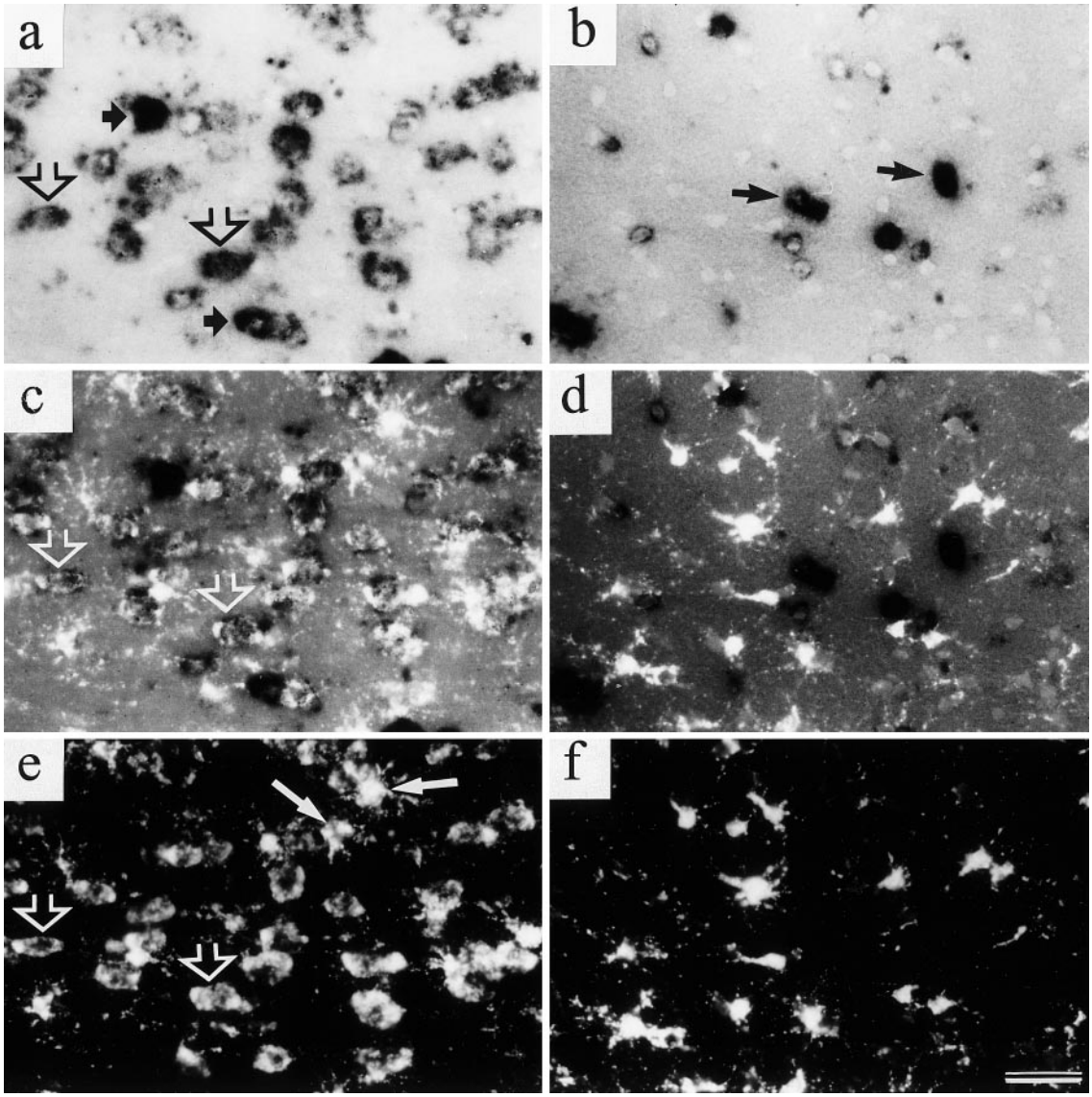

Fig. 4 Higher magnification of EAAC1 and GLT1 labeling in the deep part of layer VI of the cortex (**a, c, e**) and corpus callosum (**b, d, f**). Double-hybridized sections: **a, b** (EAAC1) and **e, f** (GLT1) show the single signals; **c, d** show both signals simultaneously. **a** EAAC1 labeling is present at moderate intensity in the majority of neurons; a few neurons are strongly labeled (*small arrows*). **c** Simultaneous EAAC1 and GLT1 labeling in neurons; *open arrows* depict examples of neurons that are double-labeled for EAAC1 and GLT1. **e** GLT1 labeling is present in neurons and astrocytes (*filled arrows* show two examples of astrocytes). **b** Strongly labeled EAAC1-positive cells in the corpus callosum (*arrows*). **d** Simultanous EAAC1 and GLT1 labeling, showing that EAAC1-positive cells and GLT1-positive astrocytes are separate. **f** GLT1 labeling of astrocytes. *Bar* 40 μm

Corpus callosum

A relatively high number of EAAC1-positive cells was observed in the corpus callosum (Figs. 1A, 4B). These cells were generally relatively strongly labeled and appeared to be mostly round and have not been described so far. Examination of sections double-labeled for EAAC1 and GLT1 at higher magnification indicates that the EAAC1-positive cells are not astrocytes (Fig. 4B, D, F). Based on their relatively scattered distribution in the corpus callosum, these cells are also not mature oligodendrocytes, which are mostly arranged in little strings of cells. Finally, double-labeling experiments with the microglia marker antibody OX-42 (Serotec) suggested that the EAAC1-positive cells are also not microglia (data not shown). Thus, these EAAC1-expressing cells in the corpus callosum are either neurons or a subpopulation of oligodendrocytes such as oligodendrocyte-progenitor cells. Other white matter areas like the fimbria fornix, anterior commissure or the white matter in the spinal cord also contained these EAAC1-positive cells (see below).

The distribution pattern of GLT1 and GLAST in astrocytes was identical in the corpus callosum and other white matter regions. For both GLT1 and GLAST, the expression levels in white matter astrocytes are lower than in grey matter astrocytes (Fig. 3).

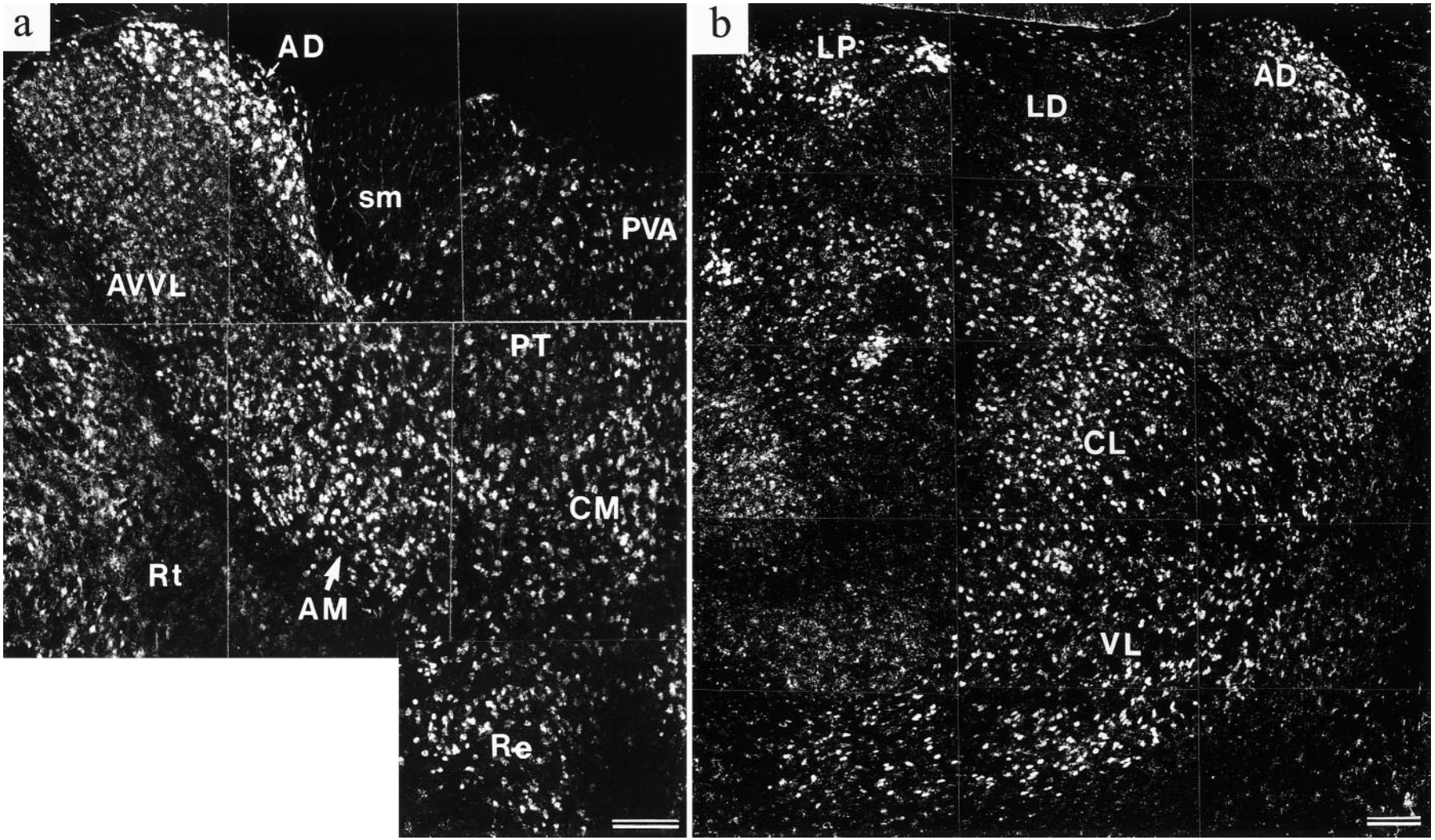

Fig. 5 Neuronal GLT1 labeling in a coronal (**a**) and sagittal (**b**) section of the thalamus. The sections were co-hybridized with GLAST probe; thus the astrocytic GLT1 labeling is blocked (except for white matter areas like the stria medullaris (*sm*) where the lower GLAST levels prevented a complete blocking of the GLT1 signal). GLT1 is relatively strongly expressed in the anterodorsal (*AD*) nucleus. Other nuclei showing neuronal GLT1 expression include: the anteromedial (*AM*), ventrolateral portion of the anteroventral (*AVVL*), posterior paraventricular (*PVA*), paratenial (*PT*), centromedial (*CM*) and centrolateral (*CL*), reuniens (*Re*), reticular (*Rt*), ventrolateral (*VL*), and the mediorostral portion of the lateral posterior (*LP*). Note that few neurons in the laterodorsal nucleus (*LD*) are labeled. *Bars* 200 μm

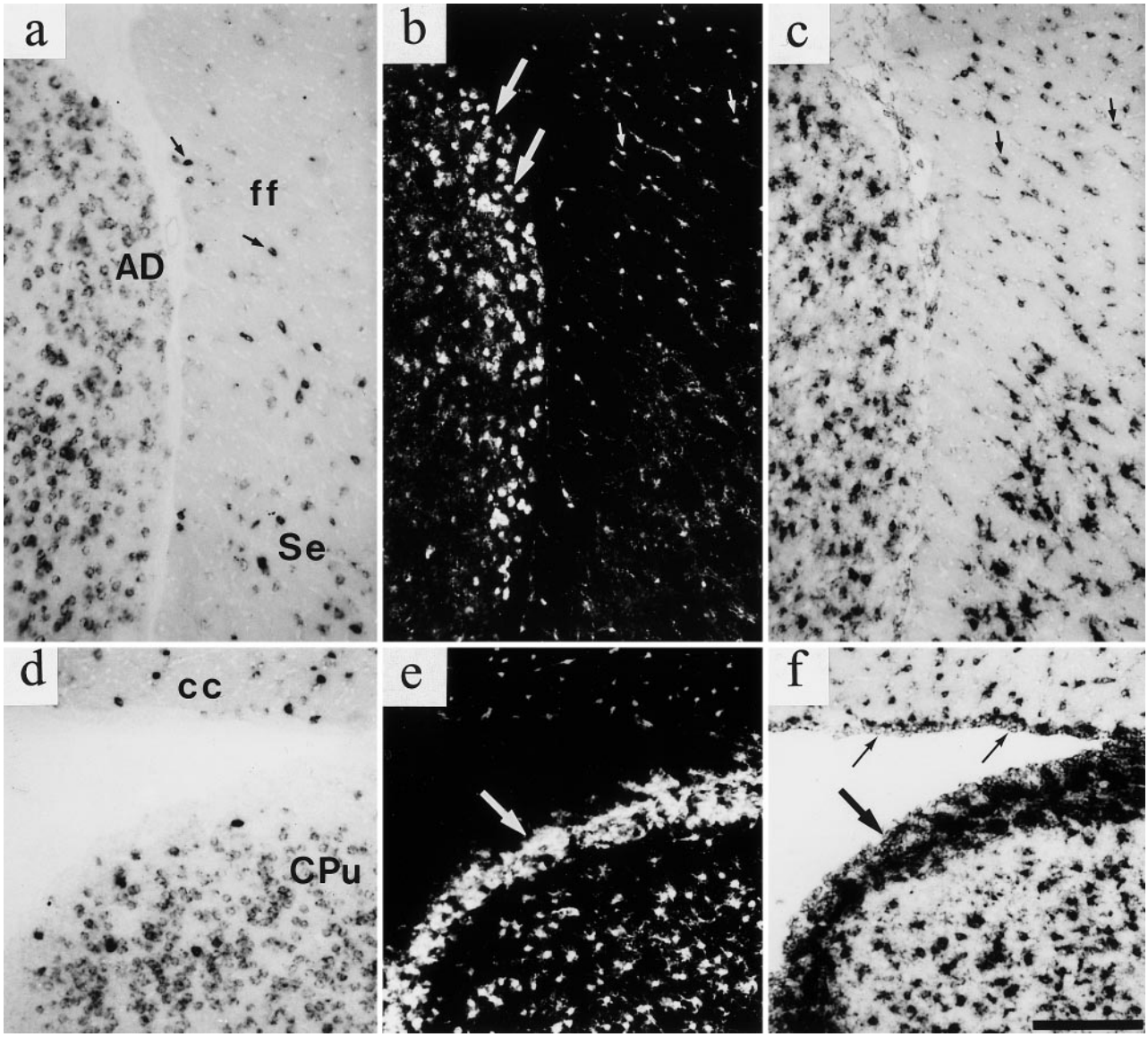

Fig. 6 Expression of EAAC1, GLT1 and GLAST in the anterodorsal nucleus of the thalamus, fimbria-fornix, and septum (**a, b, c**) and the subventricular zone in the caudate putamen (**d, e, f**). **a** EAAC1 is expressed in neurons throughout the anterodorsal (*AD*) and the adjacent anteroventral nuclei, and EAAC1-positive cells are also present in the fimbria-fornix (*ff*) and the septum (*Se*). **b** GLT1 labeling in an adjacent section co-hybridized with GLAST probe. Strong neuronal labeling is present in the anterodorsal nucleus (*big arrows*). Astrocytic GLT1 is obscured in the thalamus and septum, but not in the fimbria-fornix (*small arrows*), where GLAST labeling is weaker. **c** GLAST mRNA is strongly expressed in astrocytes of the thalamus and the septum, and only moderately in the fimbria-fornix. **d, e** Show the labeling of a section that was co-hybridized to EAAC1 and GLT1. **d** EAAC1 mRNA is moderately expressed in the small neurons in the caudate putamen (*CPu*). Note the strongly labeled cells in the corpus callosum (*cc*). **e** GLT1 labeling is present in astrocytes in CPu and cc, as well as in the subventricular plexus near the lateral ventricle (*arrow*). **f** GLAST labeling in an adjacent section. GLAST is expressed by astrocytes in CPu, cc, and the subventricular plexus, as well as by the ependymal cells lining the ventricle (*small arrows*). *Bar* 200 μm

Thalamus and subventricular zone

EAAC1 mRNA is expressed at relatively high intensity in the majority of thalamic nuclei (Fig. 1), in agreement with previous studies (Kanai et al. 1995; Torp et al. 1997). GLT1 mRNA is expressed by astrocytes and by a subpopulation of neurons in the thalamus. Previously, a neuronal cell group faintly stained for GLT1 has been observed in the medioventral thalamus (Schmitt et al. 1996). The recent report by Torp et al. (1997) mentioned a number of GLT1-positive perikarya in the thalamus, but did not identify these cells as neuronal. Using our differential double-labeling procedure, we can demonstrate the previously unrecognized large extent of the neuronal GLT1 expression in the thalamus. Figure 5A, B depicts neuronal GLT1 labeling in coronal and sagittal sections through the thalamus at approximately –1.4 mm to Bregma (Plate 24, Paxinos and Watson 1986), and 1.4 mm lateral (Plate 80), respectively. GLT1-positive nuclei include: the anterodorsal, antero-

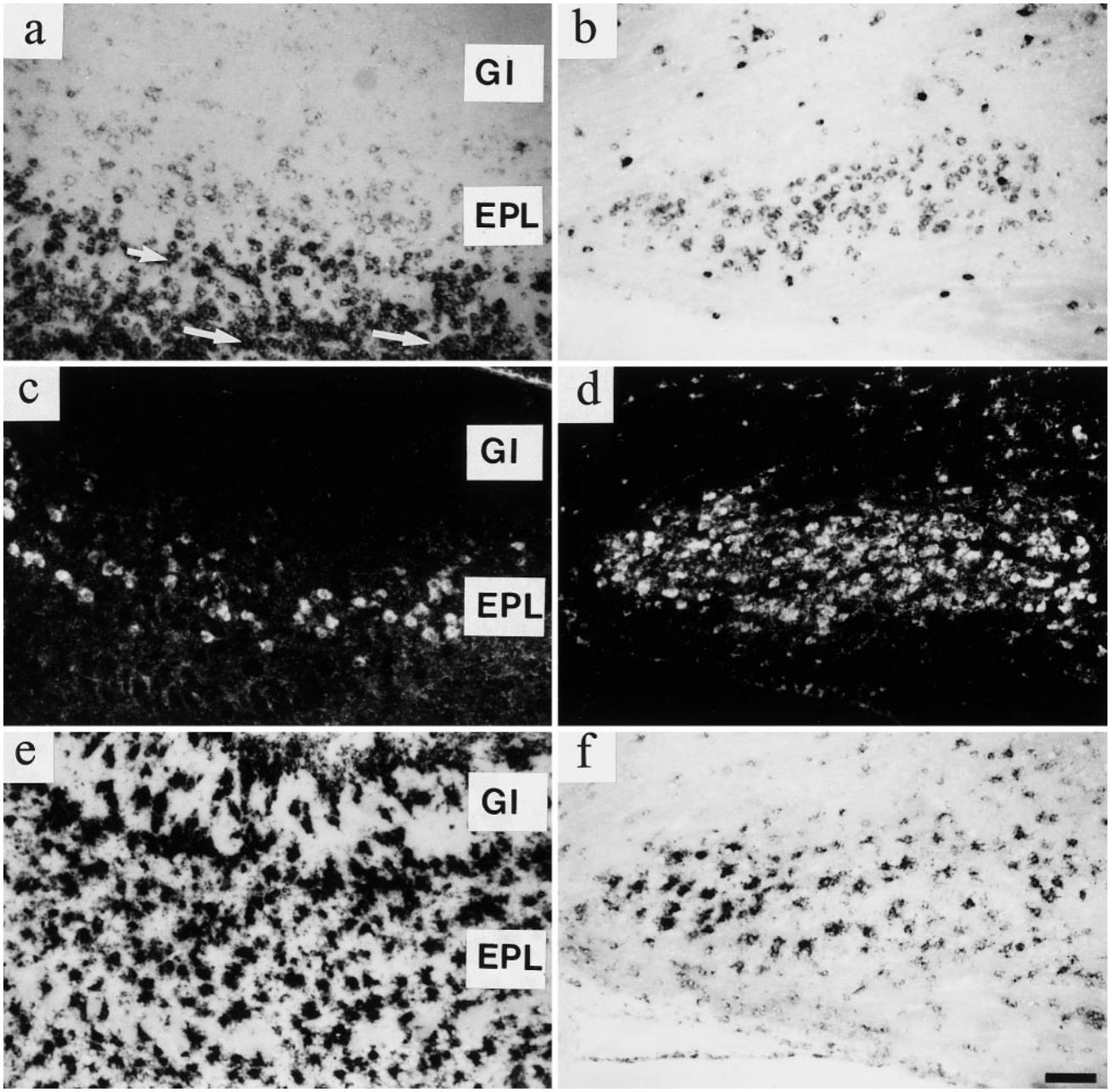

Fig. 7 Expression of EAAC1, GLT1 and GLAST in the olfactory bulb (**a, c, e**) and inferior colliculus (**b, d, f**). **c, e** and **d, f** Show GLT1 and GLAST labeling of a double-hybridized section, respectively. **a** Strong EAAC1 labeling is present in the granular (*long arrow*) and mitral cells (*short arrow*); moderate labeling is present in neurons of the external plexiform layer (*EPL*), and very weak labeling is seen in neurons in the glomerular cell layer (*Gl*). **c** GLT1 mRNA is present in neurons in the external plexiform layer; the astrocytic GLT1 labeling is obscured by the GLAST signal. **e** GLAST mRNA is strongly expressed in astrocytes of the olfactory bulb. **b** Moderate EAAC1 mRNA expression by neurons of the inferior olive. Note the strongly stained cells in the area surrounding the olive. **d** GLT1 mRNA is expressed by most neurons of this cell group. Again, astrocytic GLT1 is obscured. **f** GLAST labeling of astrocytes is relatively intense within the olive, but fainter in the surrounding areas. *Bar* 100 μm

medial, the ventrolateral portion of anteroventral, anterior and posterior paraventricular, paratenial, centromedial and centrolateral, reuniens, reticular, ventrolateral, intermediodorsal, posteromedian, and the mediorostral portion of the lateral posterior. Based on examination of coronal sections through the thalamus, GLT1 tends to be expressed by more rostrally and medially situated cell groups than by caudal and lateral groups. However, relatively strong labeling was also observed in the supragenic-ulate and the dorsal and marginal zone of the medial geniculate nuclei in the midbrain. The anterodorsal nucleus showed the highest labeling intensity (Figs. 5, 6B). No neuronal GLT1 labeling was found in several thalamic nuclei, including parafascicular, mediodorsal, and dorsomedial and ventrolateral portions of the laterodorsal nucleus.

The hybridization patterns in the anterodorsal nucleus and the fimbria-fornix/septum complex is shown for EAAC1 in Fig. 6A, for GLT1 (double-hybridized with GLAST) in Fig. 6B, and for GLAST in Fig. 6C. EAAC1 is expressed in neurons relatively evenly throughout the anterodorsal and the adjacent anteroventral nuclei and EAAC1-positive cells are also present in the fimbria-fornix and the septum (Fig. 6A). In contrast, GLT1 mRNA is relatively strongly expressed in neurons only in the anterodorsal nucleus (Fig. 6B). GLT1 mRNA is also present in astrocytes throughout this area (not visible since it is blocked by the GLAST signal). GLAST mRNA is expressed strongly in astrocytes of the thalamus and the septum, and only moderately in the fimbria-fornix (Fig. 6C).

The hybridization patterns in the lateral ventricle and underlying subventricular zone area is shown for EAAC1 in Fig. 6D, for GLT1 (co-hybridized with EAAC1) in Fig. 6E, and for GLAST in Fig. 6F. EAAC1 is widely expressed at moderate intensity in striatal neurons (Fig. 6D); strongly labeled cells are seen in the overlying corpus callosum but no labeling is present in ependymal cells or the subventricular zone (large arrow in Fig. 6E). GLT1 and GLAST are both expressed in astrocytes in the caudate putamen, and in the astrocytes forming the subventricular zone (Fig. 6E, F). No neuronal GLT1 labeling is present in this region. GLAST, but not GLT1, is additionally expressed by the ependymal cells lining the ventricle (small arrows in Fig. 6F).

Olfactory bulb and inferior olive

Figure 7A, C, E and B, D, F show the hybridization patterns for EAAC1, neuronal GLT1 (co-hybridized with GLAST), and GLAST in the olfactory bulb and the inferior olive, respectively. EAAC1 mRNA is expressed at high levels in the mitral and granule cells and at lower levels in the glomerular and external plexiform layers of the olfactory bulb (Fig. 7A). In the olfactory tubercle, EAAC1 mRNA is present in neurons throughout the anterior olfactory nucleus (Fig. 1A). GLT1 mRNA is present in astrocytes in the bulb at relatively low levels compared to either the cerebral cortex or the olfactory tubercle (Fig. 1B). However, GLT1 mRNA is also present in neurons in the external plexiform layer (Fig. 7C, double-hybridized with GLAST). Scattered GLT1-expressing neurons were also observed in the ventral and dorsal part of the anterior olfactory nucleus in the olfactory tubercle (not shown). Again, all GLT1 expressing neurons also expressed at least a small amount of EAAC1 mRNA (not shown). GLAST mRNA is expressed relatively evenly at levels equal or higher than those of cerebral cortex in astrocytes throughout olfactory bulb and tubercle (Figs. 1C, 7E).

EAAC1 mRNA is expressed at moderate levels in neurons of the inferior olive and at high levels in scattered neurons in the surrounding area (Fig. 7B). GLT1 mRNA is expressed strongly in inferior olivary neurons (Fig. 7D). In addition, GLT1 is present at moderate intensity in the astrocytes throughout this region (not shown). GLAST mRNA is expressed at high levels in astrocytes within the inferior olive (Fig. 7F), but at relatively lower levels in the area surrounding it. This strong expression of GLAST within a particular nucleus is not unique to this region and was observed in other areas including the superficial gray layer of the superior colliculus, and the trigeminal, vestibular, amygdaloid and pontine nuclei, among others (see below and Schmitt et al. 1996).

Hippocampus and locus ceruleus

The hybridization patterns in the hippocampus and locus ceruleus, as detected by single label hybridization is shown in Fig. 8A, C, E and B, D, F, respectively. EAAC1 mRNA is prominently expressed in the pyramidal neurons of CA1–3 and in the dentate gyrus granule cells, as well as in interneurons scattered throughout the molecular layer of dentate gyrus and the strata radiatum and oriens of hippocampus (Fig. 8A). GLT1 and GLAST show an almost identical expression in astrocytes throughout this region (Fig. 8C, E). One distinct difference between GLT1 and GLAST is that GLT1 is also expressed by CA3 pyramidal and deep hilar neurons, as was observed previously (Torp et al. 1994; Schmitt et al. 1996; Torp et al. 1997). In addition, relatively weak GLT1 labeling was consistently found surrounding the dentate gyrus granule cells (Fig. 8C). This granule cell labeling may represent actual labeling of astrocyte processes surrounding the granule cells, or it may reflect remaining background labeling.

EAAC1 mRNA expression is not restricted to glutamatergic or GABAergic neurons. Moderate levels of EAAC1 labeling are present in aminergic nuclei such as the locus ceruleus (Fig. 8B) or the serotonin nuclei (not shown) in the midbrain as well as in cholinergic motor nuclei in the spinal cord (not shown, but see Meister et al. 1993). GLT1 mRNA is expressed at relatively low levels in astrocytes in the locus ceruleus (Fig. 8D), and at higher levels in the astrocytes of the adjacent areas. In contrast, and similar to the inferior olive, GLAST mRNA appeared to be selectively expressed at higher levels in astrocytes of the locus ceruleus than in astrocytes surrounding this nucleus (Fig. 8F).

Hypothalamus and caudate putamen/globus pallidus

The paraventricular nucleus of the hypothalamus also shows a selective expression pattern for the three transporters. Fig. 9A, B, C shows single labeling for EAAC1, GLT1 and GLAST in this region. EAAC1 mRNA is expressed at low to moderate levels in the neurons forming the paraventricular nucleus. In the areas surrounding this nucleus, levels of EAAC1 are generally low, but a select subgroup of scattered neurons can again be identified

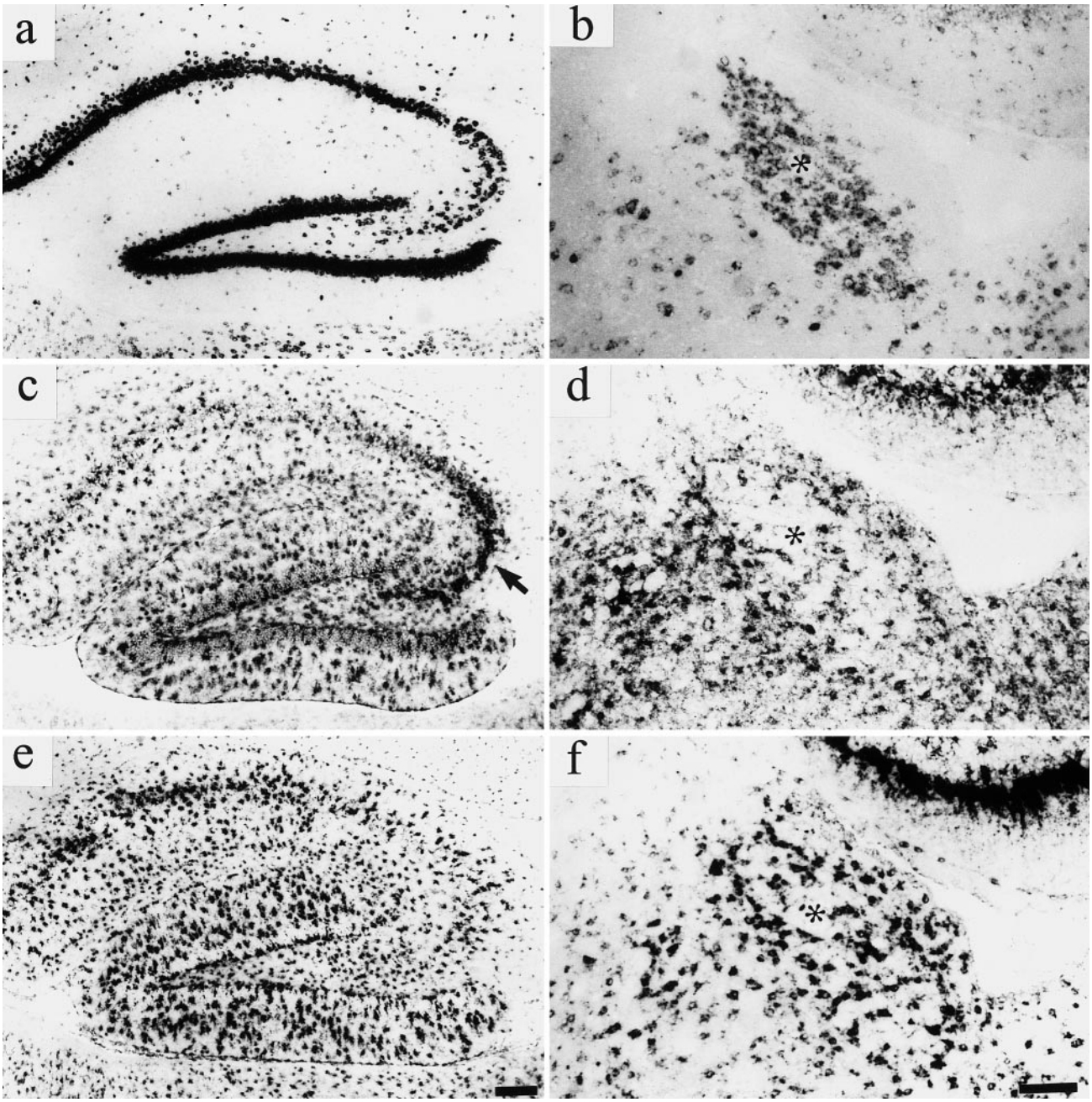

Fig. 8 Expression of EAAC1, GLT1, and GLAST in the hippocampus (**a, c, e**) and locus ceruleus (**b, d, f**). **a** EAAC1 mRNA is expressed by pyramidal cells of CA1–3, by dentate gyrus granule cells, and by neurons scattered throughout all hippocampal layers. **c** GLT1 mRNA is expressed by astrocytes and by pyramidal cells in CA3 subfield (*arrow*) and deep hilar neurons. **e** GLAST is expressed strongly by most astrocytes. **b** EAAC1 mRNA is moderately expressed by the neurons in the locus ceruleus (*asterisk*). **d** GLT1 mRNA is expressed relatively weakly by astrocytes in the locus ceruleus, and moderately by astrocytes in the surrounding areas. **f** GLAST mRNA is expressed relatively strongly in astrocytes within the locus ceruleus, and only moderately in astrocytes surrounding this nucleus. *Bars* 100 μm

that is strongly stained (Fig. 9A). GLT1 mRNA is relatively homogeneously expressed in astrocytes within the paraventricular nucleus and in astrocytes immediately surrounding it (Fig. 9B). A somewhat higher intensity of GLT1 labeling is present in the ventral areas of the hypothalamus. In contrast, GLAST mRNA is expressed in astrocytes at higher levels within the paraventricular nucleus than in the areas surrounding it (Fig. 9C).

The expression of EAAC1, GLT1 and GLAST in the globus pallidus, as detected by single labeling, is shown in Fig. 9D, E, F. The labeling intensity of EAAC1-positive neurons in the globus pallidus is similar to that in the caudate putamen (Fig. 9D). In contrast, the expression levels of both GLT1 and GLAST are markedly lower in the globus pallidus than in the adjacent caudate putamen

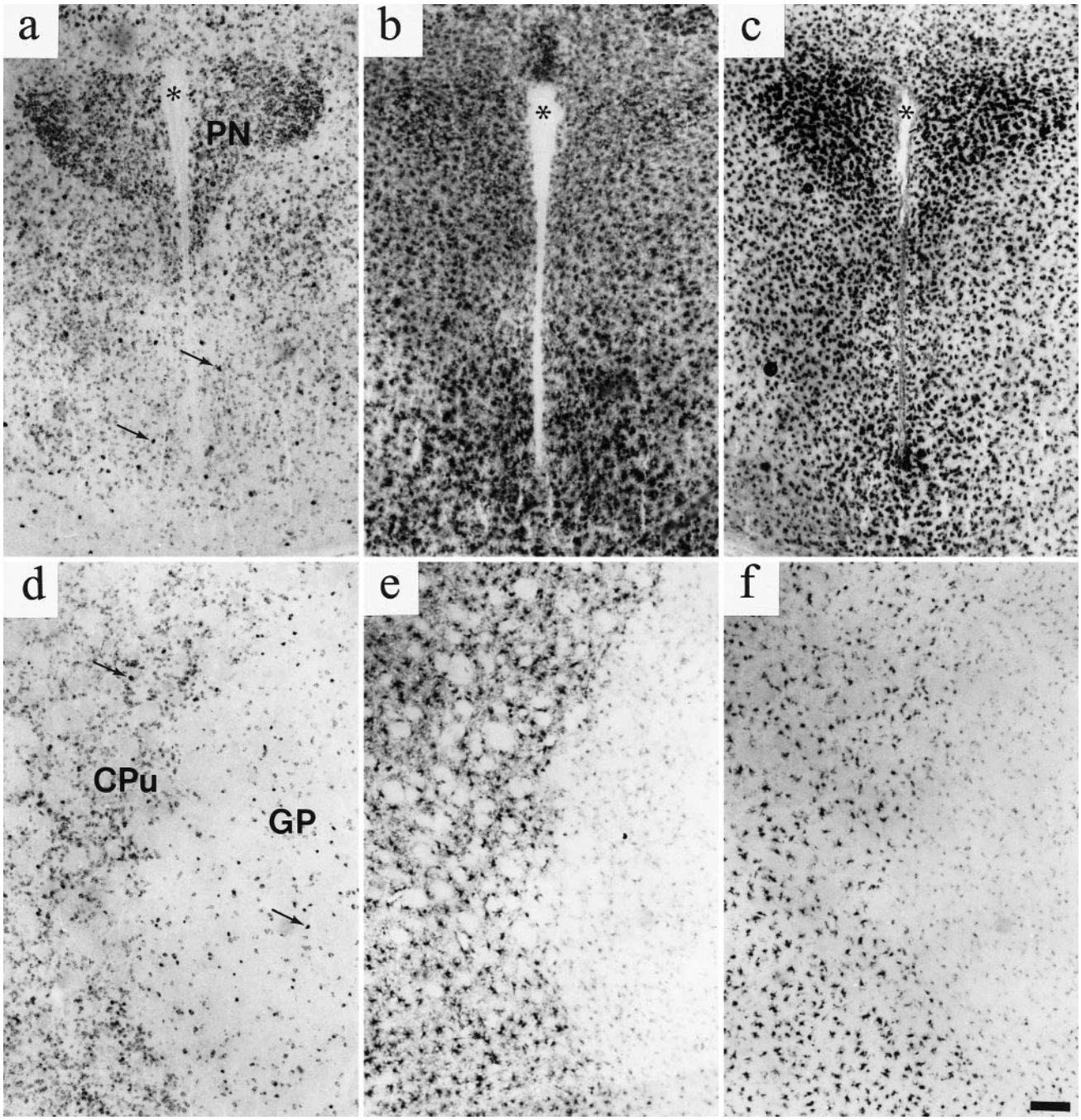

Fig. 9 Expression of EAAC1, GLT1 and GLAST in the paraventricular nucleus (*PN*) of the hypothalamus (**a, b, c**) and the caudate putamen (*CPu*) and globus pallidus (*GP*; **d, e, f**). **a** EAAC1 is expressed moderately by neurons forming the PN, and by other neurons in the hypothalamus. There is a subgroup of strongly expressing neurons that is scattered throughout this area (*arrows*). *Asterisk* indicates the third ventricle. **b** GLT1 is expressed moderately by astrocytes in PN and the areas immediately surrounding it. A somewhat stronger labeling is present in ventral areas of the hypothalamus. Based on analysis of double-hybridized sections, no GLT1 mRNA is expressed in neurons in this area. **c** GLAST is expressed strongly by astrocytes within the PN, and more moderately in other astrocytes of the hypothalamus. **d** Moderate EAAC1 expression by neurons in CPu and GP. Note the subgroup of scattered neurons that express high levels of EAAC1 (*arrows*). **e, f** GLT1 and GLAST are expressed at higher levels in astrocytes in CPu than in astrocytes in GP. *Bar* 200 μm

(Fig. 9E, F). This region, and the adjacent ventral pallidum (not shown), are the only regions next to white matter areas where GLT1 and GLAST labeling intensity was below average.

EAAC1 mRNA in cerebellum, superior colliculus, caudate putamen and anterior commissure

As shown in Fig. 10A, moderate levels of EAAC1 mRNA are contained in the granule cells and the Purkinje cells of the cerebellum. Interestingly, EAAC1 expression in Purkinje cells is more prominent in more ventrally situated cerebellar lobes (large arrow in Fig. 10A)

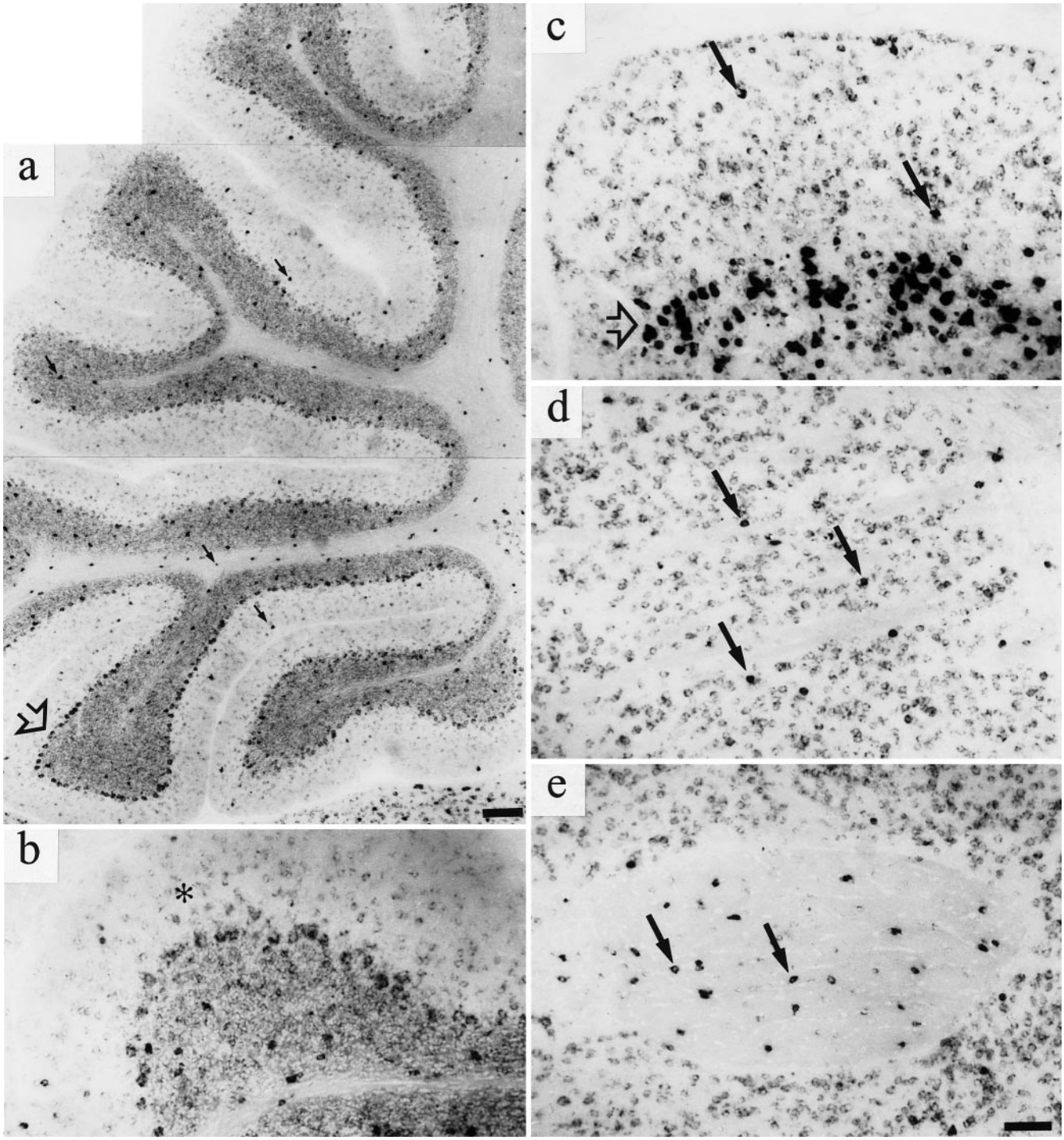

Fig. 10 Expression of EAAC1 mRNA in the cerebellum (**a, b**), superior colliculus (**c**), caudate putamen (**d**), and anterior commissure (**e**). **a** EAAC1 is moderately expressed by the granule cells, and some Purkinje cells. Note that Purkinje cells in ventral lobes (*open arrow*) are more strongly labeled than in dorsal lobes. Subgroups of scattered cells express EAAC1 very strongly (*arrows*). **b** Neurons in the molecular layer of the cerebellum (*asterisk*) are also weakly labeled for EAAC1. **c** In the superior colliculus, EAAC1 is strongly expressed by neurons in the intermediate grey layer (*open arrow*) and weakly in more superficial layers. *Closed arrows* point to isolated strongly expressing cells in superficial layers. **d** EAAC1 is expressed moderately in most striatal neurons, and strongly in scattered neurons (*arrows*). **e** Strongly EAAC1-expressing cells are present in the anterior commissure, as in other tracts of white matter. *Bars* **a** 200 μm, **b–e** 100 μm

than in dorsal ones. Faint EAAC1 labeling is also present in neurons of the molecular layer (Fig. 10A, B). Groups of scattered, strongly EAAC-positive neurons can also be identified; they are present in white matter as well as in the granular and molecular layers (small arrows in Fig. 10A, B). As previously reported, in Bergmann glia GLAST expression is very high while GLT1 expression is only moderate (see Fig. 1). Astrocytes in granular and white matter layers express moderate levels of both GLT1 and GLAST (not shown).

Subgroups of scattered neurons that express high levels of EAAC1 message are also found in grey matter areas such as the inferior colliculus or the caudate putamen (Fig. 10C, D) or white matter bundles such as the anterior commissure (Fig. 10E). Figure 10C also shows the band of the previously identified, strongly expressing neurons in the intermediate gray layer of the superior colliculus. EAAC1 is weakly expressed also by the majority of neurons in the superficial gray layer of the superior colliculus.

Discussion

This study compares the mRNA expression pattern in the brain of the three widely distributed glutamate transporters EAAC1, GLT1 and GLAST. In general, the results obtained with our improved hybridization method corroborate and extend previous results obtained with less sensitive methods. Our single and double-in situ hybridization protocols allowed a more precise analysis of the cellular localization of these transporters and revealed the following important observations. First, the expression of GLT1 message by neurons is more widespread than previously reported. Second, the expression of GLT1 and GLAST by astrocytes appears to be differentially regulated at the mRNA level depending on the brain area. Third, the EAAC1 expression by neurons is more widespread than previously thought and displays a remarkable heterogeneity with regard to the message levels present.

Specificity of single- and double-label hybridization

The specificity of our in situ hybridization labeling methods is confirmed by the unique signal patterns observed for each of the three transporters, and by the absence of any labeling when sense probes were used. The labeling patterns observed agree with the patterns observed in previous studies (Storck et al. 1992; Torp et al. 1994, 1997; Kanai et al. 1995; Kiryu et al. 1995; Schmitt et al. 1996, 1997; Sutherland et al. 1996; Velaz-Faircloth et al. 1996). The incorporation of the biotin-tyramide signal amplification made it possible to visualize GLT1 mRNA at the fluorescent level. This in turn allowed the analysis of the co-localization of GLT1 and EAAC1, or GLT1 and GLAST in the same cell, using the alkaline phosphatase-NBT/BCIP reaction to detect either EAAC1 or GLAST and the fluorescent label to detect GLT1. Preadsorption experiments ensured that the GLT1 probe does not cross-hybridize with either the EAAC1 or the GLAST probes. Furthermore, the localization of GLT1 mRNA using fluorescent detection was identical to that seen with alkaline phosphatase-NBT/BCIP detection.

Expression of GLT1 mRNA by neurons

A unique feature of our double-labeling technique is that, if development conditions are chosen so that the NBT/BCIP precipitate of the first RNA probe is very dense in a given cell, it can block either the access of the fluorescent marker for the second RNA probe or prevent its photic excitation and emission, thereby canceling its signal. We exploited this feature in our study to determine the extent of GLT1 mRNA expression in neurons. By combining hybridization for GLAST with GLT1, we were able to selectively label the GLT1 expressing neurons with a previously unachieved clarity. Earlier studies have indicated the expression of GLT1 in neurons of the cerebral cortex layer VI, medioventral thalamus, hippocampal CA3 and the dorsal endopiriform nucleus (Torp et al. 1994; Schmidt et al. 1996; Torp et al. 1997). We have now confirmed these findings and extended them to also include other nuclei in thalamus, neurons in layers II to VI of the neocortex, neurons in the inferior olivary nucleus, neurons in the external plexiform layer of the olfactory bulb, and neurons in the anterior olfactory nucleus. Further, our double-in situ procedure has also demonstrated that the majority of neurons that express GLT1 message also express EAAC1 mRNA, at least to some extent. Extensive immunocytochemical studies have so far failed to demonstrate the presence of GLT1 protein in neurons of normal neonatal or adult brain (Chaudhry et al. 1995; Lehre et al. 1995; Furuta et al. 1997; Ullensvang et al. 1997). It is possible that the GLT1 protein in neurons may be modified after translation in a way that would preclude detection by antibodies. Alternatively, the GLT1 mRNA may only be translated into protein under special circumstances (e.g. neuronal injuries, see below). In any event, it is not known why only selective neurons in the cortex, thalamus, anterior olfactory nucleus, olfactory bulb, hippocampus and inferior olive express GLT1 message and not all neurons.

Recent studies suggest that GLT1 expression by neurons may be developmentally regulated: GLT1 protein is expressed by neurons in fetal brain of sheep (Northington et al. 1997), and by neurons in primary cultures of neonatal hippocampus (Dhond et al. 1997). Furthermore, GLT1 protein expression has recently been found in neurons after hypoxic-ischemic insult in newborn striatum (Martin et al. 1997). Thus, neurons may have the general capability to express GLT1 protein, but only under certain conditions. In this context, it is interesting to note that bipolar neurons in adult normal retina have been found to express GLT1 protein (Rauen and Kanner 1994).

Differential expression of GLT1 and GLAST message

Our study directly compares the mRNA expression of GLT1 and GLAST using sensitive non-radioactive in situ hybridization. Previous studies have either compared the two expression patterns with a radioactive method, which has lower cellular resolution (Torp et al. 1994; Sutherland et al. 1996), or they studied them separately (Schmitt et al. 1996, 1997). Studies using immunocytochemistry at the light and electron microscope levels have shown that in select subregions of the brain (hippocampus, cerebellum, corpus callosum) astrocytes express both transporter proteins simultaneously (Chaudhry et al. 1995; Lehre et al. 1995; Haugeto et al. 1996). Our double-label results directly confirm, at the mRNA level, that simultaneous expression of GLT1 and GLAST occurs in the majority of astrocytes in the brain. Furthermore, our results show that there exist marked differences in mRNA expression levels in astrocytes between GLT1 and GLAST not only in Bergmann glia of the cerebellum, as previously noted (Torp et al. 1994), but also in a range of other brain regions, including the olfactory bulb, paraventricular nucleus of the hypothalamus, inferior olive, inferior colliculus, locus ceruleus, and cranial nerve nuclei such as trigeminal, or vestibular nuclei. In most of these regions, GLAST mRNA expression is relatively strong compared to the surrounding areas, while GLT1 mRNA expression is more evenly distributed. There are also areas where GLT1 expression is strong and GLAST expression is moderate, such as the inferior colliculus, or where GLT1 expression is moderate and GLAST expression is low, such as the white matter. Finally, in the globus pallidus and ventral pallidum, expression for both GLT1 and GLAST is below average. The selective high levels of GLAST mRNA in certain brain areas have also been noted by Schmitt et al. (1997). These differential expression patterns of GLT1 and GLAST suggest different regulatory pathways. Consistent with this, recent in vitro data show that expression of GLT1 requires the presence of neuronal factors while that of GLAST does not (Gegelashvili et al. 1997; Swanson et al. 1997). Furthermore, GLT1 and GLAST show marked differences in their developmental expression patterns (Shibata et al. 1996; Furuta et al. 1997; Ullensvang et al. 1997). Comparison of our results with an immunocytochemical study of the distribution of GLT1 and GLAST protein (Lehre et al. 1995) suggests that, at least macroscopically, the differences in mRNA levels correspond to differences at the protein level. For example, GLAST protein is very high in Bergmann glia compared to other brain areas, or GLT1 protein is low in the olfactory bulb compared to the hippocampus (Lehre et al. 1995). Further, areas receiving primary afferents in the brainstem and the globus pallidus/ventral pallidum area show differences in protein labeling intensities that correlate with the differences in mRNA levels observed in the present study. However, there is also evidence that glutamate transporter mRNA and protein levels do not always correlate. Studies with cultured astrocytes have shown that glutamate and kainate significantly up-regulate the expression of GLAST protein but not of its mRNA (Gegelashvili et al. 1996). In any event, our findings support the concept that GLT1 and GLAST are regulated differentially, depending on cell type and environmental cues (Gegelashvili and Schousboe 1997). Differential regulation may also be the result of different signalling pathways and the number and type of glutamatergic input projections.

Distribution of EAAC1 mRNA

The improved sensitivity of our protocol revealed several important new aspects of the distribution of EAAC1-expressing neurons in the brain. First, strongly stained EAAC1-positive cells are present in considerable amounts in white matter tracts such as the corpus callosum, fimbria-fornix, anterior commissure, or optic nerve. To our knowledge such a population of EAAC1-expressing cells has not been described so far, either at the mRNA or protein level. This cell population was also labeled in previous studies that had used radioactive or non-radioactive hybridization methods, but it had not been identified, possibly due to reduced sensitivity and/or reduced cellular resolution of the procedures used (Kanai et al. 1995; Kiryu et al. 1995; Torp et al. 1997). These EAAC1-expressing cells do not co-localize either with GLT1 or GLAST message, or with the microglia marker OX-42, and based on their distribution they do not appear to be mature oligodendrocytes. It is possible, that these cells represent a subpopulation of oligodendrocytes such as oligodendrocyte progenitor cells. Alternatively, these cells may be neurons. Interestingly, expression of EAAC1 has recently been described in cultured rat oligodendrocytes (Wang et al. 1997). The second new aspect is that subgroups of cells were identified that expressed EAAC1 at relatively high levels, and that appeared to be scattered throughout the brain, particularly in the cortex, striatum, hypothalamus, midbrain and cerebellum. The identity of these subgroups, which may include the EAAC1-positive cells in white matter tracts, is also unknown. In the cerebellum, the strongly EAAC1-positive cells in the granular layer are probably Golgi neurons, while those in the molecular layer are likely to be stellate and basket cells. Attempts to correlate the “scattered” EAAC-1 expressing cells with NADPH-diaphorase labeling for nitric oxide synthase were unsuccessful (data not shown). We hypothesize that these cells are metabolically very active and have a high turnover rate of their glutamate transporters, and therefore require higher EAAC1 mRNA levels. Finally, while our results confirm the EAAC1 expression by glutamatergic neurons such as the pyramidal cells in the hippocampus and cerebral cortex, our sensitive detection method has revealed that the majority of neurons in the brain express at least some amount of EAAC1, including the aminergic neurons in locus ceruleus, GABAergic neurons in the striatum and cerebellum (Purkinje and Golgi neurons and

stellate and basket cells) or cholinergic motor neurons in the spinal cord. This widespread expression of EAAC1 may be related to the role of glutamate as an important intermediate in cell metabolism and as a precursor for GABA synthesis.

The expression of EAAC1 in Purkinje cells in the cerebellum was quite varied, with cells in more ventral lobes expressing higher levels than those in dorsal lobes. This differential expression was observed in at least five different animals and stands in contrast to immunocytochemical studies that have found no such gradient in EAAC1-positive Purkinje cells (Rothstein et al. 1994). A second example of such a "mismatch" between mRNA and protein levels is found in the neurons of the intermediate gray layer of the inferior colliculus. Several studies have observed very high mRNA levels in these cells (Kanai et al. 1995; Torp et al. 1997), but immunocytochemical analyses have not found similarly high protein levels in these neurons (Rothstein et al. 1994; Furuta et al. 1997). Clearly, further studies are needed to elucidate the mechanisms that regulate EAAC1 mRNA and protein levels in the brain.

Conclusions

The distribution of the glutamate transporter expression observed in our study correlates well with the proposed roles of glutamate as the predominant excitatory transmitter, a precursor for GABA synthesis, and a central intermediate of amino acid metabolism. Thus, the mRNA levels for EAAC1, GLT1 and GLAST are particularly high in the neocortex and hippocampus, two brain regions in which glutamate is the main transmitter in the intrinsic and efferent projections. Further, EAAC1 is moderately expressed by GABAergic neurons such as the projection neurons in the caudate putamen or the Purkinje cells in the cerebellum. Also, low levels of EAAC1 expression were found in the majority of neurons, which may relate to the role of glutamate as a metabolic intermediate.

Using our novel double-in situ labeling procedure, we were able to unequivocally demonstrate, and map the extent of, the expression of GLT1 message in neurons. Furthermore, the double-labeling experiments revealed that GLT1 and GLAST are co-expressed by most astrocytes in the CNS, even though to varying degrees. Finally, our results demonstrate a remarkable heterogeneity in the mRNA expression levels for EAAC1 in the brain.

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