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Endogenous synthesis and transport of creatine in the rat brain : An in situ hybridization study.

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Abstract

Creatine is synthesized from arginine by L-arginine:glycine amidinotransferase (AGAT) and S-adenosyl-L-methionine:N-guanidinoacetate methyltransferase (GAMT) and can be taken up by cells by creatine transporters (CRT). While creatine is mainly synthesized by the liver and the kidney, most of other tissues, including the brain, also express AGAT and GAMT. There is evidence that the permeability of the blood-brain barrier (BBB) for creatine is limited, suggesting that the brain is dependent on its own creatine synthesis. In order to better understand creatine synthesis and transport in the central nervous system (CNS), we studied the regional distribution of cells expressing AGAT, GAMT and the creatine transporter CRT1 in the adult rat brain by non-radioisotopic *in situ* hybridization. AGAT and GAMT presented an ubiquitous neuronal and glial expression, whereas CRT1 was present in neurons and oligodendrocytes throughout the brain, but not in astrocytes. This indicates that all cells in the CNS can synthesize creatine from arginine. The absence of expression of CRT1 in astrocytes and particularly in those contacting capillary endothelial cells (BBB) reinforces the idea that under normal conditions the creatine used by the brain is synthesized mainly in the CNS. Furthermore, the expression of CRT1 by neurons and oligodendrocytes indicates that creatine trafficking is possible in those brain areas of main creatine consumption.

Keywords: brain, creatine, L-Arginine:glycine amidinotransferase, S-Adenosyl-L-methionine:N-guanidinoacetate methyltransferase, creatine transporter, in situ hybridization.

Theme B: Cellular and Molecular Biology.

Topic: Gene structure and function: general.

1. Introduction

The creatine (Cr)/phosphocreatine (PCr) system is essential for the buffering and transport of high energy phosphates. In mammals, Cr is synthesized mostly in liver and kidney, transported through the blood and taken up in tissues with high energy demand by specific Cr transporters. However, the brain might primarily be dependent on its own Cr synthesis, as evidence has been presented that the permeability of the blood-brain barrier (BBB) for Cr is limited (see [41] for a review).

Cr is synthesized by a two-step mechanism involving L-arginine:glycine amidinotransferase (AGAT; EC 2.1.4.1) and S-adenosyl-L-methionine:N-guanidinoacetate methyltransferase (GAMT ; EC 2.1.1.2) (see details in Fig. 1). AGAT has been cloned in rat, pig and human [8,13], and GAMT cDNAs were isolated from rat, mouse and human [14,16,22]. The brain as well as primary astrocytes in culture are able to synthesize their own creatine [24,35]. Eventhough AGAT activity was detected in the brain [24,35,36], there has been no data on the expression and localization of the AGAT mRNA or protein. The GAMT activity has been shown in the brain [36] and characterized in a mouse neuroblastoma cell line [3]. Its mRNA is expressed in the rat brain [18], but its distribution in the various cell types of the CNS is not known.

Cr is taken up by cells by specific transporters belonging to the Na^+-dependent neurotransmitter transporter family (see [41] for a review), that have been characterized in rat [19,26], rabbit [7] and human [15,21,28]. By *in situ* hybridization (ISH), it has been shown that the rat Cr transporter CRT1 is highly expressed in the developing CNS of the embryo as

well as in the adult CNS [9,25,26]. Specifically, the CRT1 mRNA is widely expressed in the adult brain, in both neuronal and non-neuronal cells, with high levels in the myelinated tracts, cerebellar granule cells, hippocampal pyramidal cells, several brainstem nuclei and the choroid plexus. In brain derived cultured cells, the creatine uptake activity has been described in a mouse neuroblastoma cell line and in both mouse and rat astroglial cultured cells, but not in neuron enriched cultures, leading to the hypothesis that Cr transport might be an astroglial rather than a neuronal function [20].

Substantial evidence supports the function of the Cr/PCr system in brain energetic metabolism. Patients with GAMT deficiency, a recently described autosomal recessive inborn error of Cr synthesis [6,27,31-34], manifest neurological symptoms in early infancy and show a severe neurodevelopmental delay as well as extrapyramidal symptoms. Some of these symptoms are partially reversed with high doses of oral Cr supplementation [6,31].

While these facts support the prominent physiological role of Cr synthesis in CNS, data on the localization and expression of AGAT and GAMT are lacking. The aim of the present study was to examine in detail the mRNA expression of the AGAT and GAMT genes in the brain using non-radioisotopic *in situ* hybridization. In order to discriminate between transport and synthesis of Cr, we also investigated the expression of the Cr transporter CRT1. Our work uses a new combination of *in situ* hybridization with immunohistochemistry to precisely identify the cells expressing AGAT, GAMT and CRT1.

2. Materials and Methods

Cloning of CRT1, AGAT and GAMT cDNAs and synthesis of the riboprobes:

Partial cDNAs of the rat sequence CRT1 corresponding to nucleotides 901-2544 (Gene Bank accession number X66494), of AGAT (nucleotides 182-1314, Gene Bank U07971), and of GAMT (nucleotides 131-734, Gene Bank J03588) were isolated by reverse transcription-polymerase chain reaction (Titan One Tube RT-PCR, Roche Molecular Biochemicals, Basel, Switzerland) using rat kidney mRNA as template. Sequences were verified by sequencing and inserted into the EcoRI and HindIII multiple cloning sites of pBluescript II KS^- (Stratagene) using the di-/trinucleotide sticky end cloning method [4], yielding pBS-CRT1, pBS-AGAT and pBS-GAMT. Digoxigenin labelled CRT1, AGAT and GAMT riboprobes were transcribed *in vitro* as described previously [1,2]. The antisense probes were transcribed from the plasmids linearized with XhoI (pBS-CRT1) or XbaI (pBS-AGAT and pBS-GAMT), while the sense probes were synthesized from the same plasmids linearized with BamHI (pBS-CRT1), SalI (pBS-AGAT), or XhoI (pBS-GAMT).

In situ hybridization (ISH) and immunohistochemistry

Four female adult Wistar rats (300 g, BRL, Basel, Switzerland) were deeply anesthetized (10 mg/rat Ketalar, Parke Davis, USA) and sacrificed by decapitation. Their brain was extracted within 2 minutes, immediately embedded in tissue freezing medium (Jung, Nussloch, Germany) and frozen in isopentane and dry ice. Brains were kept at -80 °C until used, then cut and analyzed by ISH as described [1,2]. Briefly, cryosections (20 µm thick) were postfixed 10 min in 4% paraformaldehyde-PBS, washed 2x15 min in PBS containing 0.1%

fresh DEPC and equilibrated 15 min in 5xSSC. Sections were hybridized (58°C for 40 h in 5xSSC, 50% formamide and 40 µg/ml salmon sperm DNA) with the digoxigenin-labelled antisense and sense riboprobes (400 ng/ml) for rat CRT1, AGAT and GAMT. The sections were then washed (30 min in 2xSSC at room temperature, 1 h in 2xSSC at 65°C, 1 h in 0.1xSSC at 65°C) and stained with alkaline-phosphatase (15 h at room temperature). After staining, the sections were dehydrated and mounted (Eukitt, O.Kindler Gmbh & Co., Freiburg, Germany). The specificity of hybridization was ascertained by the use of sense probes for CRT1, AGAT and GAMT genes that have the same length, GC content and activity of digoxigenin labelling as the corresponding antisense probes. In each ISH experiment, a section hybridized with a specific antisense probe was always preceded or followed by an adjacent section hybridized with the corresponding sense control probe.

In each ISH experiment, 3 out of 4 of the antisense ISH stained sections were further processed for immunohistochemistry. On these sections, neurons, astrocytes and oligodendrocytes were labelled using monoclonal antibodies directed against Microtubule Associated Protein 2 (MAP2), Glial Fibrillary Acidic Protein (GFAP) and Myelin Basic Protein (MBP) (Roche Molecular Biochemicals, Basel, Switzerland) (Boehringer Ingelheim, Germany), respectively. After rehydration, the ISH stained sections were fixed 1 h in 4% paraformaldehyde-PBS at room temperature and washed 3x 5min in PBS. After membrane permeabilization for 5 min in 0.1% sodium citrate and 0.1% Triton X-100, sections were processed for immunohistochemistry using the Histostain-Plus kit (Zymed Laboratories). The primary antibody was diluted 1:100 in blocking solution (non-immune serum), and applied for one hour at room temperature on the sections. After washing away the primary antibody,

sections were incubated with an anti-mouse IgG biotinylated secondary antibody followed by a streptavidin-peroxidase conjugate. Peroxidase staining was performed for 10 min using aminoethyl carbazole (AEC) and H_2O_2. The double stained sections (blue signal for ISH and red signal for immunohistochemistry) were mounted in glycerol. The very low level of GAMT transcript in some areas of the brain, especially the neocortex and the white matter structures (see Fig. 2g,h and 3i), rendered the detection of GAMT mRNA in the presence of the immunohistochemical signal difficult. In those cases, the cell identification for GAMT expression was realized on adjacent sections labelled by immunohistochemistry.

Histological analysis

Sections were observed and photographed on an Olympus BX50 microscope equipped with a DP-10 digital camera (Olympus Opticals, Japan). No discrepancies was observed between the four female rats analyzed. Brain structures were identified according to Paxinos and Watson [23].

3. Results

A semi-quantitative description of AGAT, GAMT and CRT1 mRNA levels in the different brain structures is given in Table 1. The specificity of hybridization was ascertained in each case by the absence of signal obtained with the respective sense probes (Fig. 2e,j,o).

L-Arginine:glycine amidinotransferase (AGAT)

The AGAT mRNA was found in neurons, astrocytes and oligodendrocytes throughout the brain, and its cellular localization was perinuclear. Importantly, the AGAT transcript was found in the astrocytes contacting capillary and blood vessel endothelium (BBB), as shown by a co-labelling with GFAP (Fig. 2a, arrow). In the telencephalon, AGAT was expressed throughout the neocortex, in pyramidal and non-pyramidal neurons as well as in astrocytes, but for the latter at a much lower level (Fig. 2b). In the corpus callosum, AGAT was located in astrocytes (Fig. 2c, arrow) and oligodendrocytes (Fig. 2c, asterisk) as demonstrated by the co-labelling with GFAP and MPB respectively (Fig. 2c and data not shown). AGAT mRNA was also located in the putamen, globus pallidum, nucleus accumbens, pyriform cortex and its transition towards the amygdala nuclei, and was abundantly expressed in the olfactory bulb. In hippocampus, the AGAT transcript was localized at high levels in the CA1 layer and the dentate gyrus (Fig.2d, DG). The CA3, polymorphic and molecular layers presented a lower signal, including in the astrocytes (Fig.2d, arrow). Most structures of the diencephalon, midbrain, pons and medulla showed the AGAT transcript, with higher levels in the reticular nucleus of thalamus, the posterior lobe of pituitary, the superior colliculus, the red nucleus and the trapezoid body nucleus, the subcoeruleus, the nucleus of the solitary tract and the

hypoglossal nucleus. In cerebellar cortex, AGAT was abundantly expressed in neurons of the granular layer (Fig 3a-c, G). Purkinje cells (Fig. 3b-c, arrowhead) and Bergmann glia (Fig. 3c, open arrowhead) were positive for AGAT mRNA, but at a very low level of detection, as shown by a co-labelling with MAP2 and GFAP respectively. AGAT was also found in neurons of the molecular layer (Fig. 3c), as well as in the deep nuclei of the cerebellum. The AGAT mRNA was expressed in white matter structures of cerebellum, in cells identified as astrocytes (Fig.3d-e, arrows) and oligodendrocytes (Fig. 3d-e, asterisks) with a co-labelling by GFAP and MPB (Fig. 3d and 3e respectively). The AGAT transcript was also revealed in the choroid plexus and the ependymal epithelium (data not shown).

S-Adenosyl-L-methionine:N-guanidinoacetate methyltransferase (GAMT)

The GAMT mRNA was detected in neurons and glial cells throughout the brain, with particularly low levels in astrocytes. Its main cellular localization was perinuclear. However, a lower and diffuse mRNA expression in the cell processes was also observed for GAMT in the whole brain, particularly in dense fiber regions like the cortex of telencephalon (Fig. 2g). As for AGAT, the GAMT transcript was found in the astrocytes contacting capillary and blood vessel endothelia (Fig. 2f, arrow). In the telencephalon, GAMT was expressed at low levels in neurons and astrocytes of the neocortex (Fig. 2g) as well as in astrocytes (Fig. 2h, arrow) and oligodendrocytes (Fig. 2h, asterisks) of the corpus callosum. Most other parts of the telencephalon expressed GAMT, except for the anterior commissure. In the hippocampus, the GAMT transcript was localized at low to medium levels in the CA1-3 layers, whereas the dentate gyrus presented higher levels (Fig.2i). Low levels of the GAMT mRNA were found in most nuclei of the diencephalon, midbrain, pons and medulla. Higher levels were detected in

the nucleus of the solitary tract and the hypoglossal nucleus. In cerebellar cortex, GAMT was expressed in neurons of the granular layer (Fig 3f-h). Purkinje cells (Fig. 3g-h, arrowheads) and Bergmann glia (Fig. 3h, open arrowhead) were positive for GAMT at a very low level of detection, as shown by a co-labelling with MAP2 and GFAP respectively. Low levels were found in the molecular layer and in the deep nuclei of cerebellum. The GAMT mRNA was expressed in astrocytes (Fig. 3i-j, arrow) and oligodendrocytes (Fig. 3i-j, asterisks, co-labelling with MBP) of the white matter of the cerebellum. The GAMT transcript was detected at very low levels in the choroid plexus and the ependymal epithelium (data not shown).

Creatine transporter (CRT1)

Throughout the brain, the CRT1 mRNA was localized in neurons and oligodendrocytes, with a perinuclear cellular localization. However, as for GAMT, it was also observed as a diffuse signal in the cell processes throughout the brain, in dense fiber regions like the neocortex. It was never detected in astrocytes, including those contacting capillary and blood vessel endothelial cells (BBB), as illustrated with a co-labelling with GFAP (Fig. 2k, arrows). In the telencephalon, CRT1 was expressed in the neocortex, in pyramidal and non-pyramidal neurons (Fig. 2l, co-labelling with MAP2). In the corpus callosum, it was located in oligodendrocytes (Fig. 2m, asterisk) but not in astrocytes (Fig. 2m, arrows) as demonstrated with a co-labelling by GFAP and MPB (Fig. 2m and data not shown, respectively). CRT1 mRNA was located in the putamen, globus pallidum, nucleus accumbens, pyriform cortex and in the amygdala nuclei, and presented a higher level in the olfactory bulb. In the hippocampus, the CRT1 transcript was most abundant in the CA1 layer and the dentate gyrus (Fig.2n). Most structures of the diencephalon, midbrain, pons and medulla showed the CRT1

transcript, with higher levels in the posterior lobe of the pituitary, the trapezoid body and gigantocellular reticulate nuclei, the nucleus of the solitary tract and the hypoglossal nucleus. In cerebellar cortex, CRT1 was abundantly expressed in neurons of the granular layer (Fig 3k-m). Purkinje cells were positive for CRT1 mRNA with a very low level of detection (Fig. 3l-m, arrowhead), but CRT1 could not be detected in the Bergmann glia (Fig. 3m, open arrowheads). CRT1 was also found in neurons of the molecular layer and in the deep nuclei of the cerebellum. In the white matter structures of the cerebellum, the CRT1 mRNA could not be detected in astrocytes (Fig. 3n-o, arrows) but was expressed in oligodendrocytes (Fig. 3n-o, asterisks), as shown by the co-labelling with GFAP (Fig. 3n) and MBP (Fig. 3o) respectively. The CRT1 transcript was expressed in the choroid plexus and the ependymal epithelium (data not shown).

4. Discussion

4.1: Expression of AGAT, GAMT and CRT1

Although the enzymatic activity of AGAT has been detected in the brain [24,35], no data were published concerning its cellular expression. In this study, we have shown the ubiquitous expression of AGAT mRNA in the adult rat brain, namely in neurons, astrocytes and oligodendrocytes (see summary in Table 2), as well as in the ependymal epithelium and the choroid plexus. The enzymatic GAMT activity and mRNA were previously reported in the brain [18,36], but no specific cell type localization has been described so far. Our study indicates that GAMT is ubiquitously expressed in the adult rat brain, in the same cells as AGAT but at a lower transcript level. Our work suggests that every cell in the CNS appears to be able to convert L-arginine to Cr.

The CRT1 transcript has been localized in specific areas of the brain by several authors [9,25,26]. Our work is in agreement with these studies, showing expression throughout the brain in both neuronal and non-neuronal cells, with prominent hybridization signals in the neocortex, white matter structures, granule neurons of dentate gyrus and cerebellum, several brainstem nuclei and the choroid plexus. Interestingly, and due to our double-labelling experiments that allowed the identification of the CRT1 expressing cells, we were unable to localize CRT1 in astrocytes. This result is in contrast with previous data showing the CRT1 activity in astroglial cultures [20]. This could eather mean that astrocytes of the adult rat brain do not express CRT1 mRNA, or that its expression is below the limit of ISH detection, that has been estimated at 20-30 transcripts per cell with this probe length (1644 nucleotides, [2]).

In contrast, CRT1 was well expressed in oligodendrocytes, confirming its expression in the white matter described earlier [9,25]. The absence of CRT1 mRNA in astrocytes and particularly in those participating to the BBB suggests that in normal conditions the production of Cr within the brain is predominant as compared to its blood supply. In a model of brain cell aggregate cultures issued from the rat embryo neocortex [12], we have also observed that astrocytes do not express CRT1, contrary to neurons and oligodendrocytes (manuscript in preparation), confirming the *in vivo* expression of CRT1 in the brain. The discrepancy in CRT1 expression between *in vivo* (this study and [9,25,26]) and culture [20] conditions suggests that the normal expression of CRT1 *in vivo* depends on intercellular interactions.

We have shown that AGAT, GAMT and CRT1 mRNAs are localized or concentrated in the perinuclear region of the brain cells. However, we have also observed the presence of GAMT and CRT1 transcripts in the cell processes, as it was observed for CAT3 or nNOS mRNAs [1]. Thus, GAMT and CRT1 mRNAs might be transported along the cell processes, in order to allow the brain cells to translate these proteins at the site they are needed. This would allow the cell to respond rapidly to immediate peripheral needs in Cr, a process of primary importance during synaptogenesis or growth cone migration which has been shown to be coupled directly to creatine kinase (CK) or arginine kinase [39]. The anterograde transport of newly translated proteins towards cell processes is however not excluded for GAMT, CRT1 and AGAT particularly, since its transcript was shown here to be restricted to the perinuclear region.

4.2 : Creatine synthesis and transport

The total level of Cr (Cr + PCr) in the CNS seems to correlate well with the brain CK activity as well as with the expression of the Cr transporter (see [41] and references therein). In chicken and rat brains, the different isoforms of CK (Brain-CK, Muscle-CK and Mitochondrial-CK) have been attributed to specific cell types presenting high and fluctuating energy demands, particularly Bergman glial cells, Purkinje neurons and the glomerular structures of the cerebellum [10,17,37]. We have shown that AGAT, GAMT and CRT1 are expressed in these cells, with the exception of CRT1 in the Bergman glia. Important levels of PCr and total Cr have been described in the white matter structure of human and rat [38,40], in good correlation with our data on the expression of AGAT, GAMT and CRT1 in oligodendrocytes. Higher amounts of CK and PCr have been found in glial cells compared to neurons [10,11] and primary astrocytes are able to synthesize their own Cr [5]. Our work suggests thus that astrocytes may actually not only cover their own needs in Cr but also supply some but not all of the Cr needed by other cells, e.g. neurons and oligodendrocytes. The absence of CRT1 transporter in the astrocytes participating to BBB as well as the ubiquitous expression of AGAT and GAMT suggest that under normal conditions, the brain cells synthesizing Cr depend on the uptake of arginine from the blood and on its local trafficking between the different cell types. Among the different cationic amino acid transporters (CAT, system y^+) responsible for the arginine transport, CAT1 has been shown at the BBB as well as expressed ubiquitously in neuronal and glial cells of the adult rat brain, whereas CAT3 was shown restricted to neurons [1,30]. Another form of CAT, CAT2(B), could be responsible for the arginine trafficking in glial cells [29]. In summary, every brain cell would be equipped with arginine transporters and would be able to ensure its local needs in

arginine for Cr synthesis.

In patients with the recently described GAMT deficiency, a marked increase of guanidinoacetate in the brain, CSF, serum and the urine has been shown with a concomittant decrease of Cr and PCr in the brain as measured by magnetic resonance spectrometry [6,27,31-34]. Oral supplementation with Cr (4-8 g_day^{-1} or 2 g_kg^{-1}_day^{-1}) normalized the brain Cr concentration very slowly (50% of the normal level after 6 weeks, and the normal level was reached only after 25 months of treatment; [6,27,31,32]). This observation is in accordance with our data in the rat and supports the view that *in vivo* the permeability of the BBB for Cr is very limited, probably due to the absence of CRT1 expression in the astrocytes participating to the BBB.

Our data suggest that all cells in the CNS are able to synthesize their own Cr locally because of their expression of AGAT and GAMT. Moreover, neurons and oligodendrocytes seem able to take up Cr by expressing CRT1, whereas astrocytes, particularly those contacting the capillary endothelial cells (BBB), do not express CRT1. This suggests that in normal conditions Cr for the brain is mainly synthesized there. The brain could therefore be considered as a near-independent compartment for the synthesis and the use of creatine.

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Figure legends:

Figure 1: Creatine synthesis and uptake. Adomet: S-Adenosyl-L-methionine, AdoHCys: S-Adenosyl-L-homocysteine, ADP: adenosine diphosphate, AGAT: L-Arginine:glycine amidinotransferase, Arg: arginine, ATP: adenosine triphosphate, CAT: cationic amino acid transporter, CK: creatine kinase, CRT1: creatine transporter, GAMT: S-Adenosyl-L-methionine:N-guanidinoacetate methyltransferase, Gly: glycine, Orn: ornithine.

Figure 2: Expression of AGAT, GAMT and CRT1 mRNA in the telencephalon. a-d: ISH antisense staining for AGAT, co-labelled by immunohistochemistry for GFAP (**a,c,d**) and MAP2 (**b**). **e**: ISH sense staining for AGAT. **f-i**: ISH antisense staining for GAMT, co-labelled by immunohistochemistry for GFAP (**f**). **j**: ISH sense staining for GAMT. **k-n**: ISH antisense staining for CRT1, co-labelled by immunohistochemistry for GFAP (**k,m**) and MAP2 (**l**). **o**: ISH sense staining for CRT1. **a,f,k**: Blood-brain barrier with astrocytes (**arrows**) extending their podocytes towards capillaries. **b,g,l**: Layer V of neocortex (pyramidal neurons: **arrowheads**; diffuse expression of GAMT in cell processes: **g, open arrowhead**). **c,h,m**: White matter of corpus callosum (astrocytes: **arrows**; oligodendrocytes: **asterisks**). **d,e,i,j,n,o**: Dendate gyrus (**DG**) and hilus (**H**) of hippocampus. Bar: 50 µm.

Figure 3: Expression of AGAT, GAMT and CRT1 mRNA in the cerebellum. a-e: ISH antisense staining for AGAT, co-labelled by immunohistochemistry for MAP2 (**b**), GFAP (**c,d**) and MBP (**e**). **f-j**: ISH antisense staining for GAMT, co-labelled by immunohistochemistry for MAP2 (**g**), GFAP (**h**) and MBP (**j**). **k-o**: ISH antisense staining

for CRT1, co-labelled by immunohistochemistry for MAP2 (**l**), GFAP (**m,n**) and MBP (**o**). **a-c, f-h, k-m**: Cerebellar cortex. **b,c,g,h,l** and **m** are higher magnifications of area **1** examplified in panel **a**, showing the granule cell layer (**G**), the molecular layer (**M**), the Purkinje cells (**arrowheads**) and the Bergmann glia cells (**open arrowheads**). **d,e,i,j, n** and **o**: White matter of cerebellum with higher magnification of area **2** examplified in panel **a** (astrocytes: **arrows**; oligodendrocytes: **asterisks**). Bar: 100 µm (**a,f,k**) and 50 µm (**b-e, g-j, l-o**).

Table 1: Differential expression of GAT, GAMT and CRT1 in the adult rat brain.

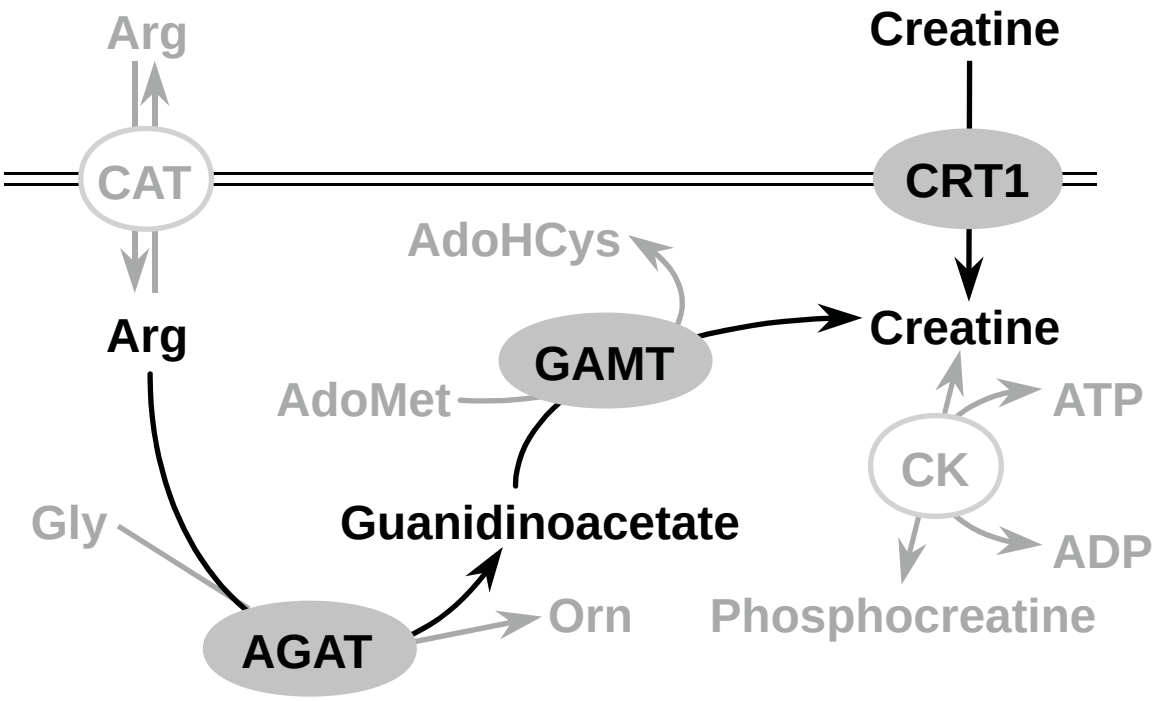
	GAT	GAMT	CRT1
Telencephalon			
Neocortex			
Pyramids of layers III and V	+	±	++
Other neurons	++	+	++
Astrocytes	±	±	-
Corpus callosum			
Astrocytes	+	+	-
Oligodendrocytes	++	+	++
Olfactory bulb	+++	+	++
Anterior olfactory nucleus	+	+	+
Anterior commissure	+	–	+
Caudate-Putamen	+	±	+
Amygdala	++	±	+
Island of Calleja	+++	+	+
Nucleus accumbens	++	+	+
Pyriform cortex	+	+	++
Hippocampus			
CA1	++	+	++
CA3	±	±	+
Dentate gyrus	+++	++	+++
Polymorph./Mol. layers			
Neurons	+	±	+
Astrocytes	+	±	-
Septal nuclei	++	±	++
Diagonal band	++	±	++
Globus Pallidum	++	+	+
Diencephalon			
Thalamus			
Stria medullaris	++	+	+
Laterodorsal nucleus	+	±	+
Lateroposterior nucleus	+	±	+
Lateroventral nucleus	+	±	+
Ventromedial nucleus	+	±	+
Posterior group nuclei	+	±	+
Anterior pretectal nucleus	+	±	+
Reticular nucleus	++	+	+
Hypothalamus			
Lateral hypothalamic area	+	±	+
Premammillary ventral nucleus	+	±	+
Zona Incerta	+	±	+
Preoptic area	+	±	+
Pituitary (posterior lobe)	++	+	++

Midbrain			
Superior colliculus	++	+	+
Inferior colliculus	+	+	±
Red nucleus	++	+	+
Substantia nigra	+	±	+
Retrorubral fields	+	±	+
Brainstem			
Pontine nucleus	+	+	+
Trapezoid body nucleus	++	+	++
Vestibular nuclei	+	±	+
Tegmental nuclei	++	+	+
Locus coeruleus	+	±	+
Subcoeruleus nuclei	++	±	+
Reticulate formation	+	+	+
Gigantocellular reticulate nuclei	+	+	++
Inferior olive	+	+	+
Trigeminal nuclei	+	+	+
Solitary tract nucleus	++	++	++
Hypoglossal nucleus	++	++	++
Cerebellum			
Molecular Layer	+	+	+
Purkinje cells	±	±	±
Bergmann glia	±	±	-
Granular layer			
Granule cells	++++	++	+++
Astrocytes	±	±	-
Cerebellar white matter			
Astrocytes	+	±	-
Oligodendrocytes	+	±	+
Deep nuclei neurons	++	±	++
Choroidal plexus	+	±	+
Ependymal and subependymal layers	±	±	+

-: absent; ±: barely detectable; +: weak expression; ++: moderate expression; +++: strong expression; ++++: very strong expression. The semi-quantitative description of AGAT, GAMT and CRT1 mRNA levels is based on ISH only experiments. Levels of transcripts observed by optical microscopy are indicated by - and + signs, which do not represent a strict linear measure of mRNA levels.

Table 2: Summary of GAT, GAMT and CRT1 expression in the rat brain.

	GAT	GAMT	CRT1
Neurons	+	+	+
Astrocytes	+	+	-
Oligodendrocytes	+	+	+



Top

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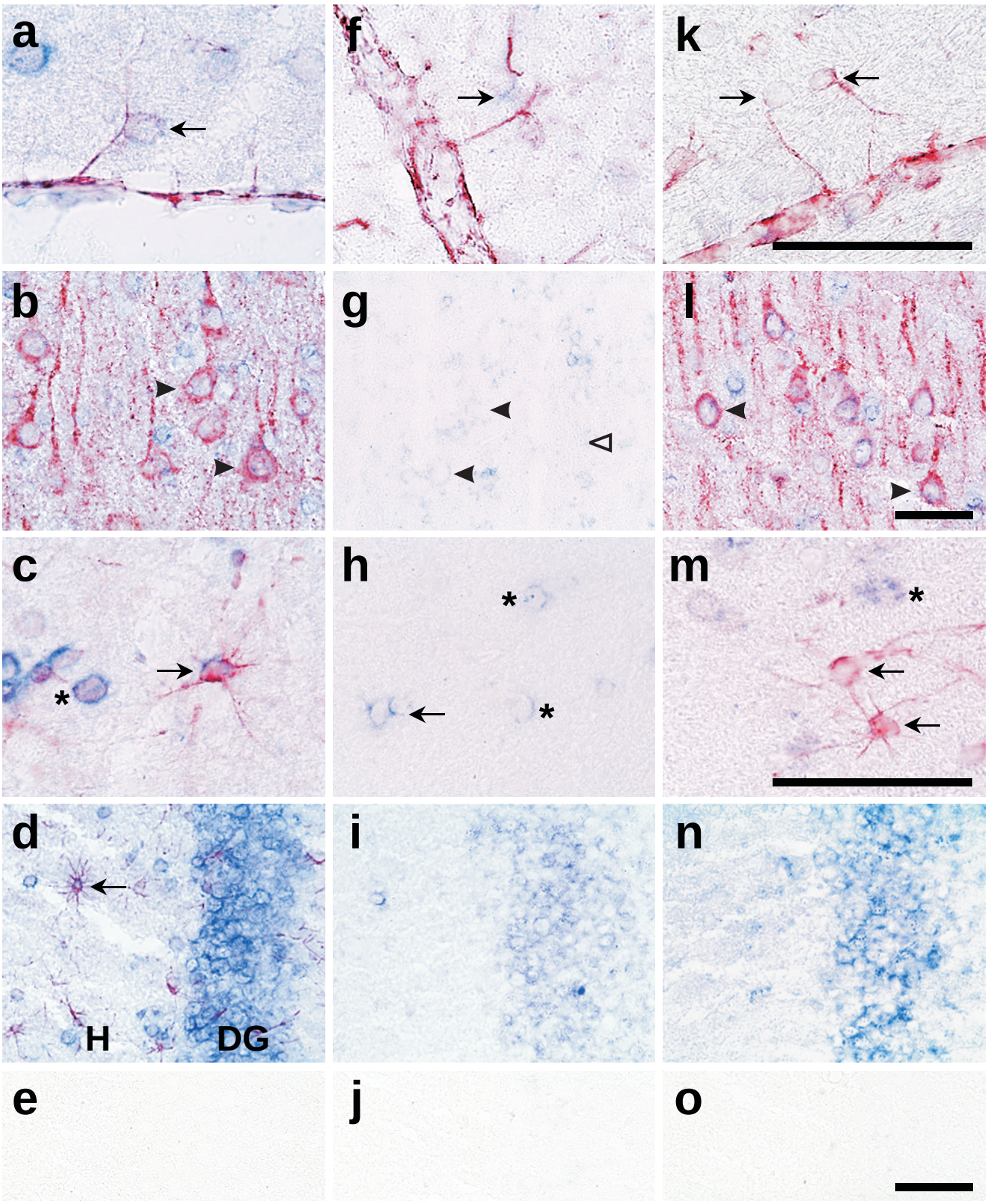

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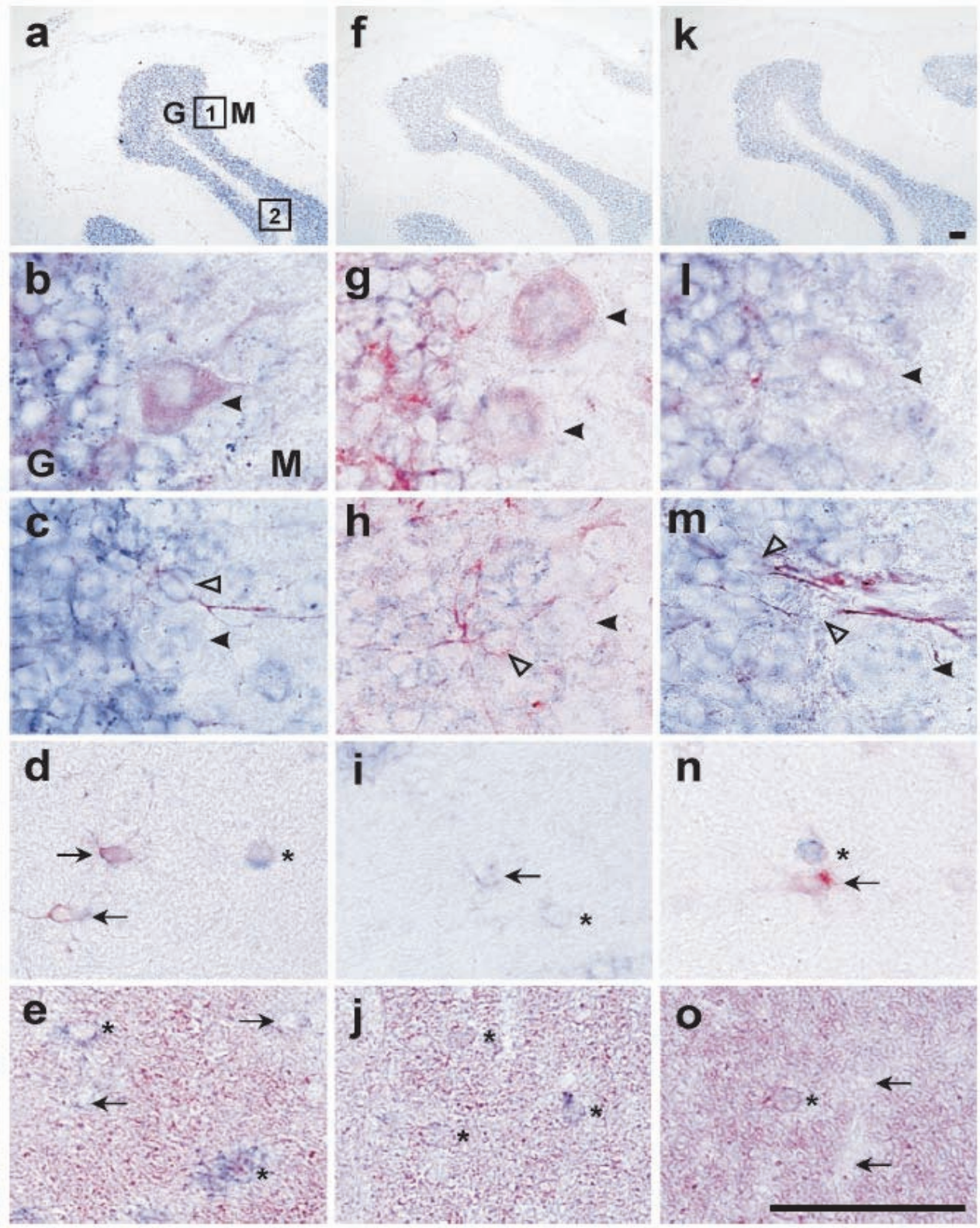

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