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Differential expression of the cationic amino acid transporter 2(B) in the adult rat brain.

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Abstract

L-arginine is a substrate for the synthesis of proteins, nitric oxide (NO), creatine, urea, proline, glutamate, polyamines and agmatine. In the central nervous system (CNS), arginine is extracted from the blood and exchanged by cells through carriers called cationic amino acid transporters (CAT) and belonging to the so-called system y^+. In order to better understand the arginine transport in CNS, we studied in detail the regional distribution of the cells expressing the CAT2(B) transcript in the adult rat brain by non-radioisotopic in situ hybridization. We show that CAT2(B) is expressed in neurons and oligodendrocytes throughout the brain, but is not detected in astrocytes. The pattern of localization of CAT2(B) in the normal adult rat brain fits closely that of CRT1, a specific creatine transporter. Our study demonstrates that the in vivo expression of CAT2(B) differs from the one reported in vitro, implying that local cellular interactions should be taken into account in studies of gene regulation of the CAT2(B) gene. Our work suggests that CAT2(B) may play a role in case of increased NO production as well as arginine or creatine deficiency in the brain.

Keywords: brain, arginine, cationic amino acid transporter, system y^+, nitric oxide, creatine, in situ hybridization.

Theme B: Cellular and Molecular Biology.

Topic: Gene structure and function: general.

1: Introduction

L-arginine is a substrate for many different pathways, including nitric oxide (NO), creatine, urea, ornithine, polyamines, glutamate, proline and agmatine, and is essential for protein synthesis [29]. In the central nervous system (CNS), arginine originates from the blood, from protein breakdown or is recycled by argininosuccinate synthetase (AS) and lyase (AL) from the citrulline generated by nitric oxide synthase (NOS).

Within the brain, specific transporters designated as system y^+ catalize the exchange of arginine between the different cell types [18]. The system y^+ was identified at the blood brain barrier (BBB) [27], in primary cultured astrocytes [24], in cultured neurons from neocortex and cerebellum [28], as well as in synaptosomes extracted from cortex and cerebellum [1]. System y^+ carriers are a family of cationic amino acid transporters called CATs [18]. Up to now, five different CAT proteins have been identified (see [19] for review): CAT1 is considered to be ubiquitously expressed except in liver [15]; CAT2(B) is present in different tissues including brain but absent from liver [17]; CAT2A, an alternatively spliced variant of CAT2(B), is liver specific [7]; CAT3 is restricted to brain and embryonic mesoderm [9,12]; finally CAT4 is expressed in placenta, testis and at a low level in brain [2,25]. CAT1, CAT2(B), CAT3 and CAT4 have been described in whole brain homogenates [9,12,15,17,25]. We and others have recently described the precise cellular distribution of CAT1 and CAT3 in the adult rat brain [5,10]. CAT2(B) was reported to be expressed at very low levels in rat primary astrocytes [26]. CAT1, CAT2(B) and CAT3 have high affinities for arginine (apparent Km around 100 µM in vitro), whereas the liver specific CAT2A has a much lower affinity (apparent Km of 2 to 5 mM). No data have been reported so far on the affinity of CAT4 for arginine.

For assessing the physiological impact of the different CAT proteins on NO production or creatine synthesis in CNS, the regulation of their gene expression is probably as relevant, if not more, as the affinity of the carriers for arginine in vitro. In order to understand the intercellular trafficking of arginine in the CNS from capillaries and their adjacent astrocytes to other glial cells and neurons, data on the detailed localization of CAT2(B) in the CNS are needed. Since these are missing, we investigated the expression of CAT2(B) mRNA in the adult rat brain. We have used a new combination of non-radioisotopic *in situ* hybridization (ISH) with immunohistochemistry to identify the cells expressing CAT2(B), and shown that it is restricted to neurons and oligodendrocytes.

2: Materials and Methods

A specific cDNA fragment for the rat CAT2 ([16]; nt 1289-1834) was obtained by reverse transcription coupled to PCR using rat liver total RNA as template, and cloned in the vector pGEM-3Zf(+) into the HincII site. The resulting plasmid was linearized with HindIII to yield the antisense probe, and with XbaI to yield the sense probe. The linearized plasmids were gel-purified and used as templates for digoxigenin labelled riboprobe synthesis, using T7- or SP6-RNA polymerases (Promega, Madison, WI), as described previously [3,5]. 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 non-radioactive ISH using the active-DEPC method as described [3,5,6]. The specificity of hybridization was ascertained by the use of a sense probe having the same length, GC content and activity of digoxigenin labelling as the corresponding antisense probe. 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), as described previously [6]. The double stained sections (blue signal for ISH and red signal for immunohistochemistry) were mounted in glycerol. Sections were observed and photographed on an Olympus BX50 microscope equipped with a DP-10 digital camera (Olympus Opticals, Japan). No discrepancies were observed between the four female rats analyzed. Brain structures were identified according to Paxinos and Watson [20].

3: Results

The CAT2(B) mRNA was found in neurons and oligodendrocytes throughout the brain, but never in astrocytes. Its main cellular localization was perinuclear. A semi-quantitative description of CAT2(B) mRNA levels in the different brain structures is given in Table 1. The specificity of hybridization was ascertained by the absence of signal obtained with the CAT2(B) sense probe (Fig.1g).

In the telencephalon, CAT2(B) was expressed in the neocortex, in pyramidal and non-pyramidal neurons (Fig.1a,b; co-labelling with MAP2). CAT2(B) was never detected in astrocytes, including those contacting capillary and blood vessel endothelial cells (BBB), as illustrated by a co-labelling with GFAP (Fig. 2d,f; arrowheads). In the hippocampus, the CAT2(B) transcript was localized at high levels in the CA1 layer and the dentate gyrus (Fig.2c). The CA3, polymorphic and molecular layers presented a lower signal. In the corpus callosum, CAT2(B) was located in oligodendrocytes as demonstrated by a co-labelling with MBP (Fig.2e). The CAT2(B) mRNA was well expressed in the olfactory bulb (Fig.1f) and also located in the putamen, the globus pallidum, the nucleus accumbens, the pyriform cortex and its transition towards the amygdala nuclei. Most structures of the diencephalon, midbrain, pons and medulla showed the CAT2(B) transcript at a weak level, except for the brainstem with higher signals in the vestibular nuclei, the locus coeruleus, the trigeminal nuclei and the solitatry tract nucleus. In the cerebellar cortex, CAT2(B) was abundantly expressed in neurons of the granular layer (Fig.2a-c). Purkinje cells were positive for CAT2(B) mRNA, but at a very low level of detection, as shown by a co-labelling with MAP2 (Fig.2b). Bergmann glial cells were negative, as shown by a co-labelling with GFAP (Fig.2c). CAT2(B) was also found in the cerebellar neurons of the molecular layer and deep nuclei. The CAT2(B) mRNA was expressed at a very low level of detection in the white matter structures of cerebellum, in cells identified as oligodendrocytes by a co-labelling with MBP (data not shown). The CAT2(B) transcript was also revealed in the choroid plexus (Fig.2a) and the ependymal epithelium (data not shown).

4: Discussion

4.1: Expression of the CAT2(B) mRNA in the brain

Previous reports have described the presence of the CAT2(B) mRNA in total homogenates of the rodent brain [17], but without a distinction of its expression among cell types. We show in this study that in normal conditions the CAT2(B) mRNA is found both in neurons and oligodendrocytes of the whole brain. However, we could never detect its expression in astrocytes, including those contacting endothelial cells of capillaries and participating to BBB. As for CAT1, the CAT2(B) mRNA was mainly localized in the perinuclear region of the brain cells; this is in contrast with the CAT3 transcript, which we have also found in the neuronal processes [5].

A comparison of the cerebral expression of the CAT2(B) mRNA with the previously described expression of CAT1 and CAT3 transcripts [5] is given in Table 2. Our studies suggest that the prime candidate for basic arginine trafficking between brain cells is CAT1, as it is ubiquitously expressed in CNS, including at the BBB [5,40]. CAT2(B) and CAT3 could be more specifically involved in the arginine uptake and exchange by neurons and oligodendrocytes (CAT2(B)) and neurons only (CAT3) (this study, and [5,10]).

4.2: Arginine uptake and metabolism in brain cells

The availability and uptake of arginine for the different types of brain cells is a prerequisite for their local NO and creatine synthesis. Evidence is growing that depending on the physiological and/or pathological conditions to which the CNS is exposed, the y^+ system of arginine transport as well as the intracellular metabolism and recycling of arginine can be regulated at the molecular level.

Rats injected intraperitoneally with lipopolysaccharide (LPS) present a dose-dependent induction of the inductible and endothelial NOS genes in the brain, which is restricted to astrocytes [13]. It was also shown that primary rat astrocytes treated by LPS and interferon-γ (IFNγ) present an induction of the CAT2(B) gene, together with an increase of their arginine uptake, an induction of the iNOS gene and an increase of their NO synthesis [26]. In the same study, primary neurons did not express CAT2(B) in control conditions, and did not induce its expression under LPS and IFNγ treatment. Neuronal PC12 cells do not express CAT2(B) either constitutively or under IFNγ and tumor

necrosis factor α (TNFα) treatment, whereas they show an induction of the iNOS gene [31]. As CAT2(B) is not expressed in vitro in primary neurons nor in PC12 cells [26,31], the constitutive neuronal expression of CAT2 we found in this study indicates that the normal brain differs from the conditions to which single cell type cultures are exposed in vitro. The CAT2(B) expression in the CNS, with its different cell types, might thus depend on intercellular interactions.

The intracellular recycling of arginine by AS and AL has also been shown to be regulated: LPS and IFNγ injected into the rat brain induce the AS gene in glial cells [8], whereas TNFα and IFNγ induce both the AS and AL genes in PC12 cells [31]. In human and mouse, primary or secondary defects of urea cycle activity lead to a significant decrease of blood arginine concentration [11,14,22,23]. This is also found in portacaval shunted or ammonium chloride-treated rats which show, in synaptosomes prepared from different parts of the brain, an increase of arginine uptake in parallel with augmented NOS activity [21]. We have recently observed that the CNS expression of the arginine recycling genes AS and AL is enhanced in astrocytes under ammonium chloride treatment [4]. Thus the arginine transport and metabolism seems affected by increased ammonia.

The arginine transport might also be relevant for the creatine synthesis in CNS. Recent studies suggest that in normal conditions the brain might depend on its own creatine synthesis due to the absence of a creatine transport system at the BBB [6,30]. In consequence, brain cells synthesizing creatine depend on the uptake of arginine from the blood and on its local trafficking between the different cell types, and on the CNS expression of the CAT genes. Interestingly, the differential pattern of expression of CAT2(B) in the rat brain closely follows the expression of CRT1, a specific transporter for creatine (see Table 2, and details in [6]). One might speculate that in the brain the expression of CAT2(B) and CRT1 is coordinated, and furthermore that CAT2(B) might be involved in the creatine synthesis pathway in the CNS.

In the non-nervous tissues, numerous factors have been reported that regulate the CAT genes at the molecular level, including cytokines, growth factors, hormones, LPS or a low protein diet (see [18,19] for reviews and references therein). In the CNS or in cell cultures issued from the nervous tissue however, only a few data are available. The CAT1 mRNA is constitutively expressed in neuronal PC12 cells as well as in primary neurons and astrocytes. However it is not regulated by

LPS, IFNγ nor TNFα treatments, nor is CAT3 induced in PC12 cells by TNFα and IFNγ [26,31]. To date in CNS, the only CAT gene which has a regulated expression appears to be CAT2(B): it is inducible in primary astrocytes under a LPS and IFNγ treatment [26]. It remains to be shown if the induction of CAT2(B) also plays a role in vivo, e.g. under pathological conditions. In particular, it could be relevant to test if the brain is able to increase its extraction of arginine from the blood by induction of the CAT2(B) gene in conditions of arginine or creatine deficiency.

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Figure legends:

Figure 1: Expression of the CAT2(B) mRNA in the telencephalon. a-f: ISH antisense staining for CAT2(B) (blue signal), co-labelled by immunohistochemistry (red signal) for MAP2 (neurons, **a,b,c**), GFAP (astrocytes, **d,f**) and MBP (oligodendrocytes, **e**). **g**: : ISH sense staining for CAT2(B). **a,b**: Layer V of neocortex (pyramidal neurons: **arrowheads**, non-pyramidal neurons: **asterisk**). **c**: Dentate gyrus of hippocampus. **d**: Blood-brain barrier in neocortex with astrocytes (**arrowheads**) extending their podocytes towards capillaries. **e**: White matter of corpus callosum (oligodendrocytes: **arrowhead**). **f,g**: Olfactory bulb. Bar: 50 µm.

Figure 2: Expression of the CAT2(B) mRNA in the cerebellar cortex. a-c: ISH antisense staining for CAT2(B) (blue signal), co-labelled by immunohistochemistry (red signal) for MAP2 (**a,b**) and GFAP (**c**). **a**: Cerebellar cortex with the molecular layer (**M**), the Purkinje cell layer (**P**) and the granule cell layer (**G**). Choroidal plexus: **asterisk**. **b,c**: Higher magnification of the area indicated in panel **a**, with Purkinje cells (**b**) and Bergman glia (**c**) located by **arrowheads**. Bar: 50 µm.

Table 1: Differential expression of the CAT2(B) mRNA in the adult rat brain.

Telencephalon		Hypothalamus (continuation)	
Neocortex		Premammillary ventral nucleus	+
Pyramids of layers III and V	++	Zona incerta	+
Other neurons	+	Preoptic area	+
Astrocytes	-	Pituitary (posterior lobe)	+
Corpus callosum			
Oligodendrocytes	+	***Midbrain***	
Astrocytes	-	Superior colliculus	+
Olfactory bulb	+++	Inferior colliculus	+
Anterior olfactory nucleus	+	Red nucleus	±
Anterior commissure	±	Substantia nigra	±
Caudate putamen	+	Retrorubral fields	±
Amygdala	+		
Island of Calleja	++	***Brainstem***	
Nucleus accumbens	++	Pontine nucleus	+
Pyriform cortex	+	Trapezoid body nucleus	+
Hippocampus		Vestibular nuclei	++
CA1	++	Tegmental nuclei	+
CA3	+	Locus coeruleus	++
Dentate gyrus	+++	Subcoeruleus nuclei	+
Polymorphic / molecular layers	+	Reticulate formation	+
Septal nuclei	+	Inferior olive	±
Diagonal band	+	Trigeminal nuclei	++
Globus pallidum	+	Solitary tract nucleus	+++
Diencephalon		***Cerebellum***	
Thalamus		Molecular layer	+
Stria medullaris	±	Purkinje cells	+
Laterodorsal nucleus	+	Bergmann glia	-
Lateroposterior nucleus	+	Granular layer neurons	++++
Lateroventral nucleus	+	Cerebellar white matter	
Ventromedial nucleus	+	Oligodendrocytes	±
Posterior group nuclei	+	Astrocytes	-
Anterior pretectal nucleus	+	Deep nuclei neurons	+
Reticular nucleus	+		
Hypothalamus		Choroidal plexus	+
Lateral hypothalamic area	+	Ependymal / subependymal layers	+

-: absent; ±: barely detectable; +: weak expression; ++: moderate expression; +++: strong expression; ++++: very strong expression. The semi-quantitative description of CAT2(B) 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 expression of CAT1, CAT2(B), CAT3 and CRT1 in the rat brain.

	CAT1	CAT2(B)	CAT3	CRT1
Neurons	+	+	+	+
Oligodendrocytes	+	+	-	+
Astrocytes	+	-	-	-

CAT: Cationic amino acid transporter; CRT1: creatine transporter. The expression data for CAT1, CAT3 and CRT1 are taken from [5,6].

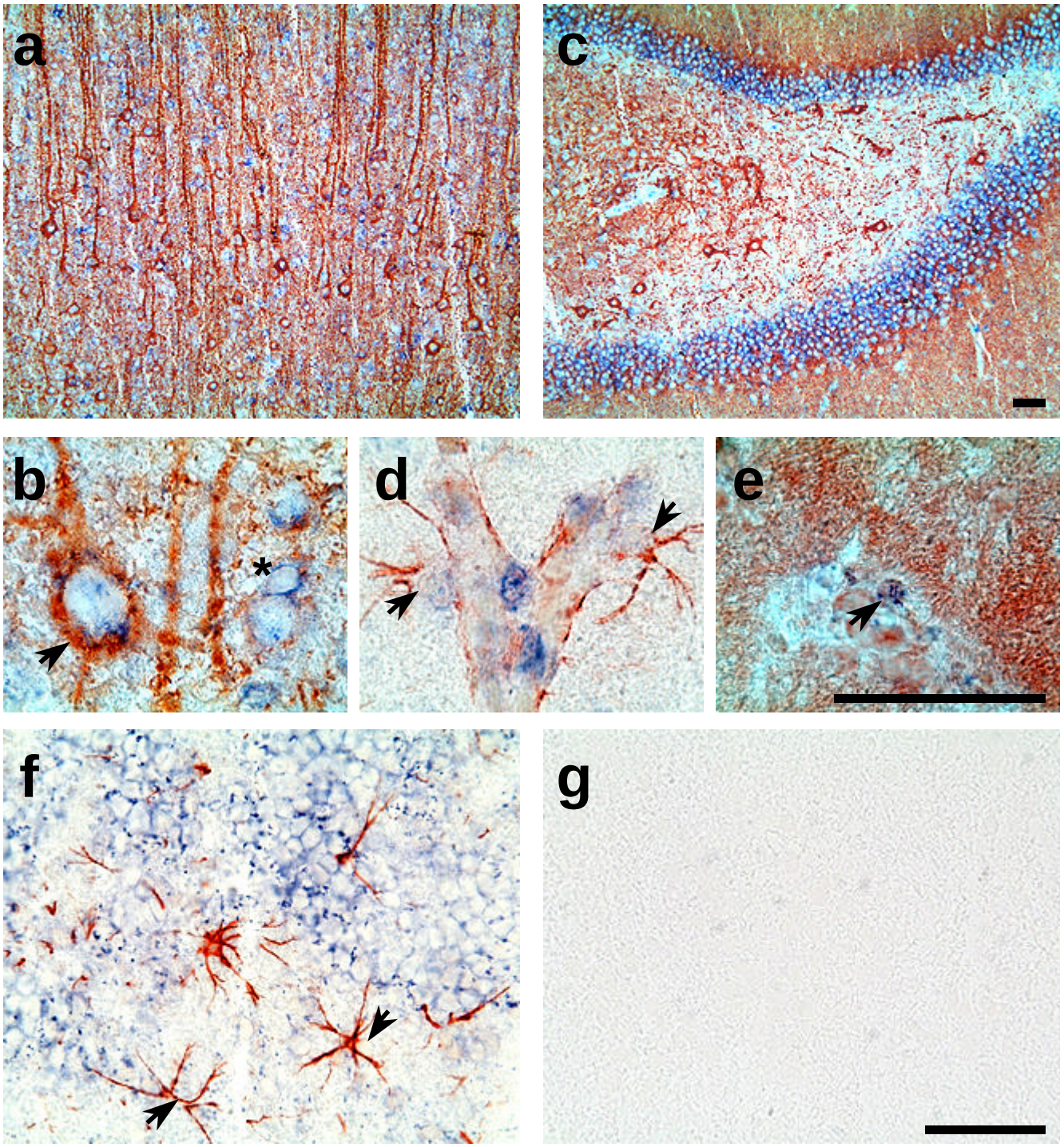

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Figure 1

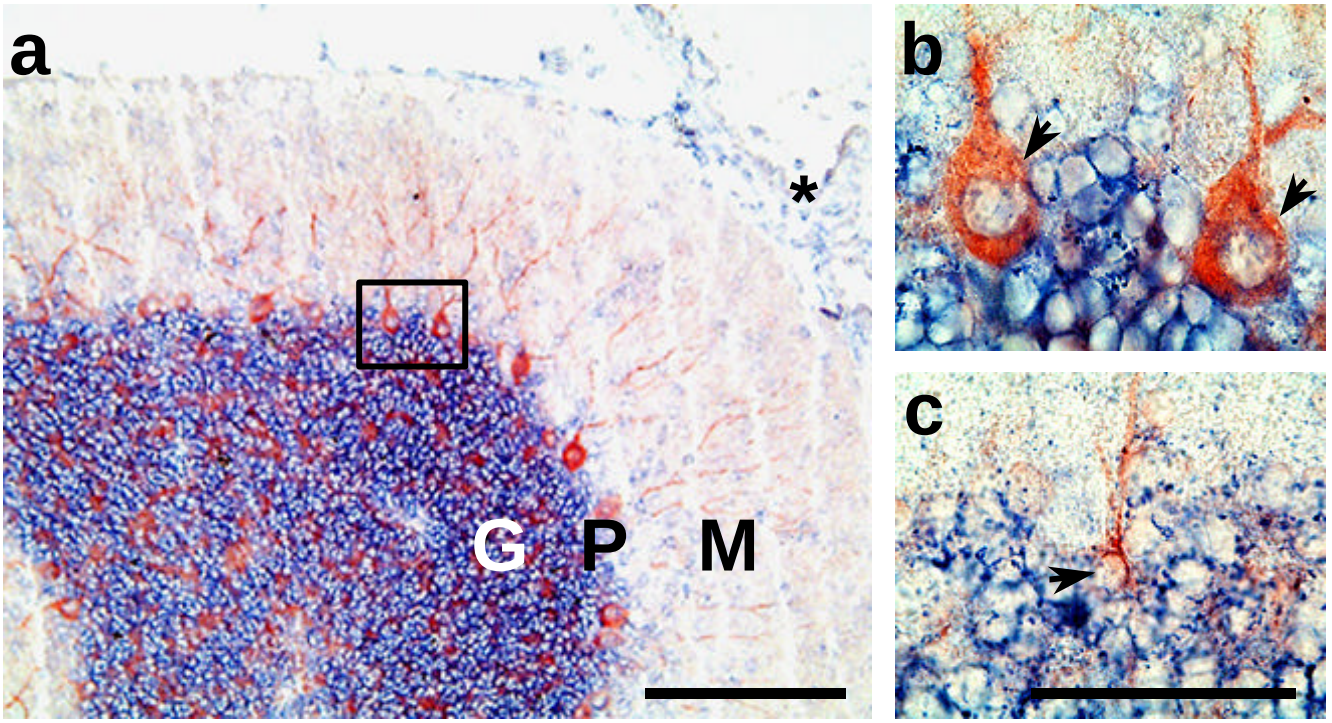

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Figure 2