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The type 2 iodothyronine deiodinase is expressed primarily in glial cells in the neonatal rat brain

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ABSTRACT Thyroid hormone plays an essential role in mammalian brain maturation and function, in large part by regulating the expression of specific neuronal genes. In this tissue, the type 2 deiodinase (D2) appears to be essential for providing adequate levels of the active thyroid hormone 3,5,3'-triiodothyronine (T3) during the developmental period. We have studied the regional and cellular localization of D2 mRNA in the brain of 15-day-old neonatal rats. D2 is expressed in the cerebral cortex, olfactory bulb, hippocampus, caudate, thalamus, hypothalamus, and cerebellum and was absent from the white matter. At the cellular level, D2 is expressed predominantly, if not exclusively, in astrocytes and in the tanycytes lining the third ventricle and present in the median eminence. These results suggest a close metabolic coupling between subsets of glial cells and neurons, whereby thyroxine is taken up from the blood and/or cerebrospinal fluid by astrocytes and tanycytes, is deiodinated to T3, and then is released for utilization by neurons.

Thyroid hormone controls a number of metabolic and developmental processes and, in particular, is an essential factor for normal mammalian brain maturation (1). In humans and other species, thyroid deficiency during the perinatal period results in irreversible brain damage and mental retardation (1, 2). The effects of thyroid hormone result primarily from changes in gene expression mediated through the binding of the active compound 3,5,3'-triiodothyronine (T3) to specific nuclear receptors of the steroid-retinoic acid-thyroid hormone superfamily. Previous studies have demonstrated that T3 nuclear receptors are expressed in a complex temporal pattern in specific regions of the brain that include the cerebral cortex, hippocampus, striatum, cerebellum, and hypothalamus (3, 4). These receptors are found predominantly in neurons and oligodendrocytes (5–7), and a number of neuronal genes have been shown to be regulated by thyroid hormone during development (8, 9).

The majority of T3 in the brain is produced locally within the central nervous system by the 5'-deiodination of thyroxine (T4) (10). The type 2 deiodinase (D2) appears to be of particular importance in catalyzing the conversion of T4 to T3 in the brain during fetal and early neonatal life (11–13). During this period in the rat, the expression of D2 activity in brain increases at the end of gestation and is highest at 15–20 days of postnatal life (14). This pattern of activity corresponds temporally to the period when the developing brain is most dependent on thyroid hormone and correlates with increasing brain T3 concentrations, which peak at 2 weeks of age (13).

An important property of the D2 is that its activity is markedly increased by thyroid hormone deficiency (15). This enhanced D2 activity serves to maintain T3 production in the

brain in the face of limiting amounts of the prohormone T4 (16). Brain T3 levels thus appear to be protected to a considerable extent by alterations in circulating thyroid hormone levels (12, 16). A second important factor in this regard is a decrease in the clearance rate of T3 in the hypothyroid brain effected by a decrease in the activity of the type 3 deiodinase (D3) (11). This enzyme converts T4 and T3 to inactive metabolites by 5-deiodination (17). The coordinated regulation of D2 and D3 activity appears to be critical for thyroid hormone homeostasis in this tissue.

Complementary DNAs for the rat and human D2 have recently been isolated and shown to code for selenoproteins, which contain the uncommon amino acid selenocysteine at the catalytic site (18). In the adult mammalian brain, Northern blot analysis indicates that D2 mRNA is expressed in the cerebral cortex, hippocampus, caudate-putamen, thalamus, and cerebellum (18). However, many important questions remain unanswered concerning the expression patterns and functional roles of the deiodinases in the central nervous system. Most notably, only limited information is available concerning the regional patterns of expression of the D2 during development, and the cell type(s) that express this important enzyme have not been identified. In the present work we demonstrate that D2 is expressed primarily in subpopulations of glial cells in thyroid hormone-dependent areas of the developing central nervous system, with the highest levels of expression found in specific regions of the hypothalamus.

METHODS

Neonatal Wistar rats (15 days old) were used in these studies. The National Institutes of Health rules and the European Union directives for the care and handling of animals were followed. Northern blots were prepared using nylon membranes (Nytran, Schleicher and Schuell), and hybridizations were carried out according to standard protocols (19, 20). *In situ* hybridization for the detection of D2 and neuronal-specific enolase mRNA was performed on free-floating 25- μ m sections using protocols previously described in detail (21). D2 sense and antisense riboprobes were synthesized in the presence of [³⁵S]UTP using a 366-bp DNA template spanning nucleotides 535–901 from the rat D2 cDNA sequence (18). This area contains little sequence homology with the rat type 1 deiodinase (D1) and D3 mRNAs. (Attempts at using digoxigenin-labeled probes in these studies resulted in unacceptable nonspecific staining.) The sections were either counterstained with Richardson's blue or subjected to immunohistochemistry for glial fibrillary acidic protein (GFAP) using a polyclonal antibody (Dako) at a 1/2,000 dilution. The atlas of Paxinos

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Abbreviations: D1, type 1 iodothyronine deiodinase; D2, type 2 iodothyronine deiodinase; D3, type 3 iodothyronine deiodinase; GFAP, glial fibrillary acidic protein; T3, 3,5,3'-triiodothyronine; T4, thyroxine.

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and Watson (22) was used for the identification of rat brain structures.

RESULTS

In situ hybridization was performed on the brains of euthyroid 15-day-old neonatal rats, the time when D2 activity is maximal in this tissue. The riboprobe used in these experiments specifically detected D2 mRNA, as demonstrated by Northern analysis, by using brown adipose tissue RNA prepared from control and cold-exposed rats (Fig. 1). Cold exposure, which is known to induce a marked increase in brown fat D2 activity (23), was associated with increased expression of a 7-kb major RNA species, as well as other minor bands, as we have previously described (18).

Representative coronal sections from different levels of the neonatal rat brain demonstrated that D2 mRNA is expressed in several regions. These include the olfactory bulb and anterior olfactory nucleus (Fig. 2*A*), the cerebral cortex (Fig. 2*A–E*), the caudate-putamen nucleus (Fig. 2*B* and *C*), the dorsal portion of the lateral septal nucleus (Fig. 2*B*), the thalamus (Fig. 2*D* and *E*), the hippocampus (Fig. 2*D* and *E*), and the hypothalamus (Fig. 2*E* and *F*). D2 was also expressed in the granular layer of the cerebellum as well as pontine nuclei such as the ventral cochlear nucleus and the olivary nuclei (Fig. 2*G*). Lower levels of expression were found in the midbrain (Fig. 2*H* and *I*). Areas of white matter, such as the corpus callosum, subcortical white matter, internal capsule, and fimbria of the hippocampus, were not labeled.

The distribution of D2 expression in the cerebral cortex was not uniform; layers 1–4 showed the strongest signal, whereas expression was low in layer 5. In the piriform cortex, expression was noted in layer 1, whereas no signal was detected in the neuronal layer 2. There was also some regionalization in the caudate, with the highest expression localized to the ventrolateral portion of the nucleus. In the hippocampus, both the dentate gyrus and the hippocampus proper (Ammon's horn) were labeled. D2 mRNA appeared to be absent from the amygdala.

An extremely high signal was present in the hypothalamus (Fig. 2*E* and *F*) in the regions corresponding to the lateral aspects of the median eminence and the lining of the lower portion of the third ventricle (Fig. 2*E* and *F*, arrows). This corresponds to the known distribution of ependymal tanycytes

(24, 25), a specialized population of glial cells implicated in the transport of hormones, and other substances in the hypothalamus (26). The relative amounts of D2 mRNA in the hypothalamus and layers 1–4 of the parietal cortex were compared by measuring the optical density of these regions on a low-exposure autoradiograph (Fig. 2*F*). Signal strength in the region of the third ventricle and median eminence was 4- to 5-fold higher than that in the outer layers of the cortex.

The expression pattern in the cerebral cortex was intriguing because layer 1, which shows high D2 expression, is practically devoid of neuronal bodies and contains mainly glial cells and neuropile. To more precisely localize the signal to specific cell types, we performed emulsion autoradiography and counterstained the sections. Fig. 3 shows corresponding dark-field and bright-field images of several brain regions. In the olfactory bulb (Fig. 3*A* and *B*), D2 was expressed in the external plexiform layer, which contains primarily glial cells and processes. The neuron-containing mitral cell and granular layers immediately above showed little signal. Also, the packed cells in the subventricular layer (Fig. 3*B*), which was still prominent at this age, completely lacked a hybridization signal. In the dentate gyrus (Fig. 3*C* and *D*), the label was present in the molecular layer, whereas the granular cell layer of neurons was practically devoid of signal. In Ammon's horn (Fig. 3*C* and *D*), the radioactive signal was present in the stratum radiatum in close association with the pyramidal cell layer. The pattern in the cerebral cortex (Fig. 3*E* and *F*) confirmed the results obtained by macroscopic autoradiography; expression was localized mainly to layers 1–4, with less signal in layers 5 and 6.

In the hypothalamus (Fig. 3*G* and *H*), high expression was present in the lining of the third ventricle (Fig. 3*G*, arrow) and in a narrow rim at the lateral and basilar regions of the median eminence (Fig. 3*G*, arrowhead). The inset in Fig. 3*H* shows a magnification of this area under bright-field illumination. The silver grains were concentrated immediately above the packed cells of the pars tuberalis (arrowheads), which completely lacked hybridization signal.

These results strongly suggested that D2 was expressed mainly by two types of glial cells, astrocytes and tanycytes. This was confirmed by performing *in situ* hybridization for D2 mRNA and immunohistochemistry with an anti-GFAP antibody on the same sections. Fig. 4*A* shows astrocytes from layer 1 of the parietal cortex. They have the typical star-like appearance, with many long and delicate processes forming an intricate network of fibers. The majority of silver grains were observed over the cell processes, sometimes at a considerable distance from the cell body. Fig. 4*B* shows colocalization of silver grains with the GFAP-positive fibers crossing the molecular layer of the dentate gyrus. The distribution of D2 mRNA in the tanycytes lining the third ventricle is shown in Fig. 4*C*. Silver grains were observed in both the cell bodies (arrows) and the cellular processes (arrowheads) that cross into the adjacent arcuate nucleus. There were also numerous hybridization grains in the apical protrusions (blebs) of the tanycytes. Although the periventricular location of the hypothalamic D2 expressing cells indicated that they were tanycytes, their identity was confirmed in other studies (data not shown) by demonstrating intense staining of these cells for vimentin, a tanycyte protein not found in mature astrocytes (27).

D2 expression in the cerebellum appeared localized to the densely packed layer of granule neurons, suggesting neuronal expression in this region. This was further examined by *in situ* hybridization using two riboprobes, an ³⁵S-labeled D2 probe and a digoxigenin-labeled neuronal-specific enolase probe, which specifically labels neurons. As shown in Fig. 4*D*, there is little association of silver grains with the neuronal-specific enolase mRNA (blue spots). In contrast, immunostaining of astrocytes with GFAP antibodies demonstrated the presence

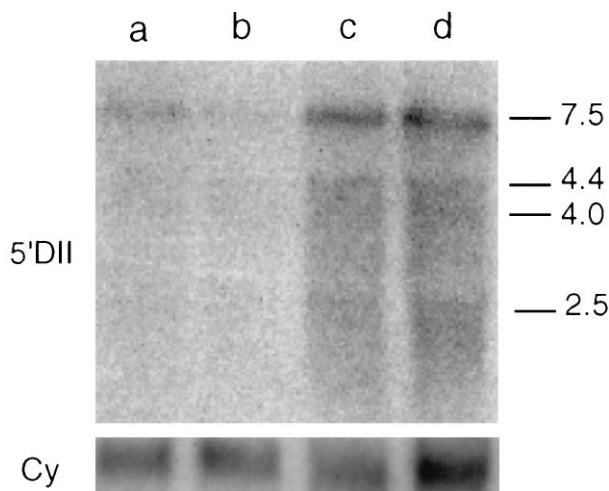


FIG. 1. Northern blot demonstrating the specificity of the cDNA template used for riboprobe preparation for detecting D2 mRNA. Each lane contained 25 μ g of total RNA isolated from the brown adipose tissue of 4-week-old control rats (lanes a and b), or rats exposed to cold (4°C) for 8 hr (lanes c and d). The position of RNA size standards in kilobases is shown. Cy, cyclophilin.

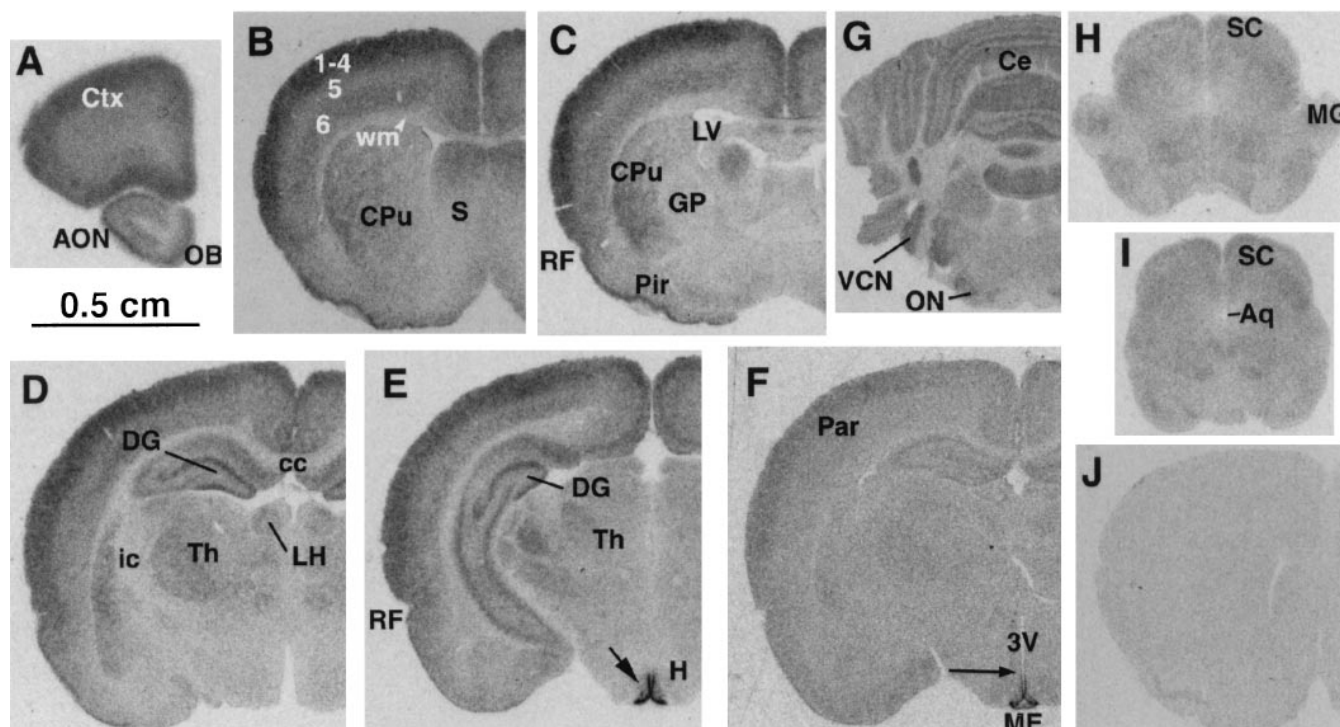


FIG. 2. *In situ* hybridization on sections of 15-day-old neonatal rat brain showing the regional distribution of D2 mRNA. All sections were taken in the coronal plane at the levels of the olfactory bulb (A); the septum (B); the globus pallidus (C); the anterior hippocampus (D); the caudal hypothalamus (E and F); the pons and cerebellum (G); and the midbrain (H) and (I). J utilized a sense probe and shows a coronal section at a level similar to that in B. All sections were exposed for 2 weeks with the exception of F, which was exposed for 1 week. The arrow in E points to a region of very high expression in the lower part of the third ventricle. The arrow in F points to the sharp transition in D2 expression occurring along the lining of the third ventricle. Aq, cerebral aqueduct; AON, anterior olfactory nucleus; Ce, cerebellum; cc, corpus callosum; CPu, caudate-putamen nucleus; Ctx, cerebral cortex; DG, dentate gyrus; GP, globus pallidus; H, hypothalamus; ic, internal capsule; LH, lateral habenula; LV, lateral ventricle; ME, median eminence; MG, medial geniculate nucleus; OB, olfactory bulb; ON, olivary nucleus; Pir, piriform cortex; RF, rhinal fissure; S, septum; SC, superior colliculus; Th, thalamus; VCN, ventral cochlear nucleus; wm, white matter. Layers of the cerebral cortex are shown in Arabic numerals.

of D2 mRNA in these cells (Fig. 4E). Thus, expression of D2 in the cerebellum is also primarily in astrocytes.

DISCUSSION

The overall pattern of D2 mRNA expression observed in these studies agrees well with prior reports describing the distribution of D2 activity in brain (28–30). Unlike these prior studies, however, which relied exclusively on the determination of 5'-deiodinase activity in homogenates of brain regions isolated by gross dissection techniques, the *in situ* studies presented here allow for the much more accurate, precise, and specific localization of D2 expression.

Of importance, D2 is expressed in areas of the brain, such as the cerebral cortex, hippocampus, caudate, and hypothalamus, which are known to express nuclear T3 receptors and to be targets of thyroid hormone action during development (3, 4). For example, congenital hypothyroidism in the rat results in marked alterations in the cytoarchitecture of the cerebral cortex (31) as well as alterations in expression of specific genes (8). The latter abnormality is also observed in the caudate and the hippocampus, where thyroid hormone controls expression of the RC3/neurogranin gene (21), whose protein product appears to be important in long-term potentiation and memory (32). Cerebral cortical and basal ganglia abnormalities are also observed in the human cretin, where mental retardation and alterations of posture and movement are observed (32). The expression of D2 in auditory relay nuclei such as the medial geniculate, the olivary, and the ventral cochlear nuclei suggests that these regions are also thyroid hormone responsive, a finding that may be relevant to the deafness observed in congenital hypothyroidism.

Previous studies using immunohistochemistry have reported that T3 receptors are located in neurons and oligodendrocytes but not in astrocytes (5–7). The finding that perinatal hypothyroidism does not affect the expression of astrocytic marker genes provides additional evidence favoring the view that astrocytes are not target cells of thyroid hormone (33). Thus, it was surprising to find that D2 is expressed primarily in specific subpopulations of glial cells, namely the protoplasmic astrocytes and tanycytes, whereas little expression appears to occur in neurons or fibrous astrocytes of the white matter. Several of our observations support this conclusion. For example, cell layers containing primarily astrocytes (e.g., layer 1 of the cerebral cortex) demonstrated strong D2 mRNA expression, whereas layers containing primarily neurons (e.g., the granular layer of the dentate gyrus) were devoid of staining (Fig. 3). Furthermore, colocalization studies demonstrated directly the presence of D2 mRNA in GFAP-positive astrocytes and tanycytes, with the majority of the signal being located in the cellular processes. This latter finding is difficult to demonstrate on some photomicrographs, given that many of the grains are located some distance from the cell bodies in delicate end processes that have been cut in cross-section and lie in different planes. However, when viewed firsthand by microscopy, which allows the processes to be traced through the 25- μ m-thick sections by altering the focus, it is readily apparent that the grains are contained within these astrocytic projections. These observations suggest a unique and important role for glial cells in thyroid hormone homeostasis in the brain.

Astrocytes serve important functions in neural biology (34, 35). Astrocytic processes ensheath synapses and thereby influence synapse formation and synaptic transmission. Glial cell

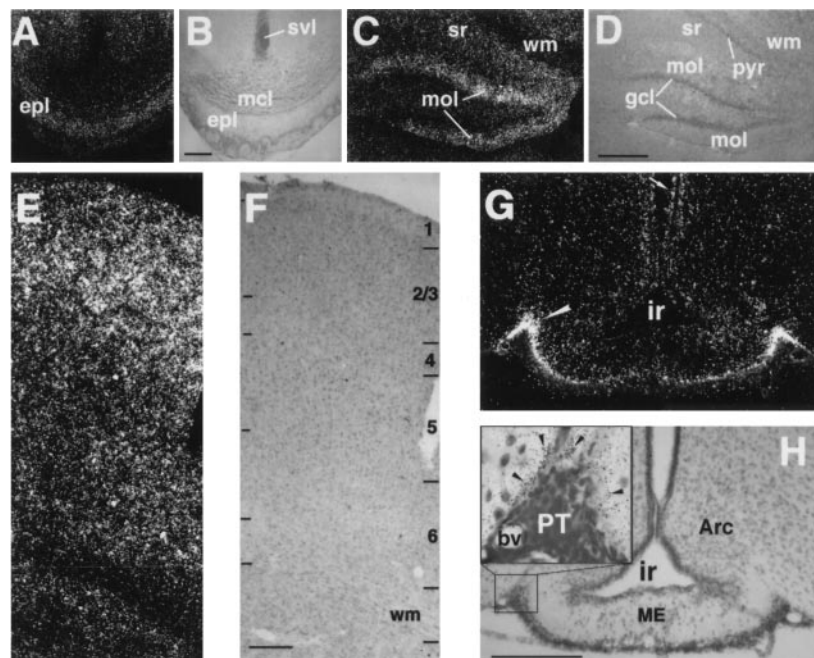


FIG. 3. Dark- and bright-field images showing the distribution of silver grains after coating the hybridized sections with photographic emulsion. The sections were exposed for 20 days. Sections shown correspond to the main olfactory bulb [dark field (A); bright field (B)], the dentate gyrus [dark field (C); bright field (D)], the somatosensory cortex [dark field (E); bright field (F)], and the hypothalamus at the level of the median eminence and arcuate nucleus [dark field (G); bright field (H)]. Arc, arcuate nucleus; bv, blood vessel; epl, external plexiform layer; Gr, granular cell layer; ir, infundibular recess; ME, median eminence; mcl, mitral cell layer; mol, molecular layer; PT, pars tuberalis; pyr, pyramidal cell layer (CA1 field); sr, stratum radiatum; svl, subventricular layer; wm, white matter. In G the arrow points to the ventricular lining and the arrowhead points to an extremely high signal above the pars tuberalis. The corresponding bright-field image is shown in the *Inset* of H, with black arrowheads pointing to the silver grains of the photographic emulsion. Photomicrographs were taken with a 5 \times objective. (Bars = 200 μ m.)

processes also surround capillaries and participate in the transfer of nutrients between the blood and neurons. For example, glucose, the main metabolic fuel used by neurons, is actually taken up from the blood by astrocytes and processed to lactate, which is then released for neuronal use (34). Glial cells also play a role in maintaining brain glutamate concentrations (36).

A similar functional coupling between astrocytes and neurons may be operative with regard to T3 production. According to this postulate, T4, which enters the developing brain more easily than T3 (12), would be taken up from the capillaries by astrocytes, deiodinated to T3, and released. This T3 would then be available to enter neurons and interact with T3 receptors to regulate specific neuronal functions.

Anatomic studies have demonstrated that both tanyocyte processes and the apical blebs that extend into the third ventricle contain ribosomes and smooth endoplasmic reticulum (24, 37). Thus, the localization of the D2 mRNA to astroglial processes and blebs suggests that this microsomal enzyme is translated and localized within these cellular compartments. Previous studies by Dratman and Crutchfield (38) support the possibility that T4-to-T3 conversion may occur within astrocytic processes, with the T3 transported to the end-feet and released. These investigators demonstrated that T3 derived from T4 in the brain is concentrated rapidly in synaptosomes in which the T3:T4 ratio is 3-fold greater than in cytosol.

The pattern of D2 mRNA in the hypothalamus strongly suggests that this enzyme is expressed primarily in ependymal cells, termed tanycytes. These cells are characterized by extensive processes that extend into all areas and cell groups of the medial hypothalamus and encircle capillaries and abut on neurons (39). Tanycytes have been implicated in the uptake and transport of hormones and other substances into the hypothalamus from the cerebral spinal fluid and the blood (40, 41). For example, the transport of T4 by ependymal cells from

the third ventricle into the median eminence and the portal vessels has been demonstrated both *in vivo* and in organ culture systems (42).

The finding of D2 expression in tanycytes suggests that these cells may not only take up T4, from the cerebrospinal fluid and/or capillaries, but also convert it to the active hormone T3 for use in various regions of the hypothalamus. This would explain the apparent paradox that the paraventricular nucleus, which contains thyrotropin-releasing hormone-secreting neurons responsive to feedback by T4, as well as T3 (43), expresses very little D2 activity (30). The deiodinating and transport capacity of tanycytes thus may represent the "alternative source of T4 monodeiodinating activity" postulated to exist by Lechan and Kakucska (44). Uptake of T4 from the cerebrospinal fluid, with its subsequent deiodination to T3 and transport to the median eminence and the portal system, could also influence pituitary function. Alternatively, T4 could be taken up from the fenestrated vessels of the median eminence, deiodinated, and the T3 transported to the cerebrospinal fluid. Indeed, bidirectional transport of substances by tanycytes between the third ventricle and the vascular compartment has been demonstrated (45).

To date, studies localizing D1 and D3 expression to specific cell types in the brain have not been reported. The D1 is also a 5'-deiodinase that converts T4 to T3 and, if present in neurons, could be an alternative source of T3 for these cells. However, only limited information is available in the literature on the pattern of 5'-deiodinase expression in the developing rat brain (14, 29), and the techniques employed in these studies have not allowed the accurate determination of D1 activity. However, ongoing studies have demonstrated that D1 activity, though detectable, is minimal in the cerebral cortex of neonatal rats (J. Bates and V. A. Galton, personal communication). This observation, along with the known poor efficiency with which D1 utilizes T4 (as compared with reverse T3) as a substrate for 5'-deiodination, makes it unlikely that this iso-

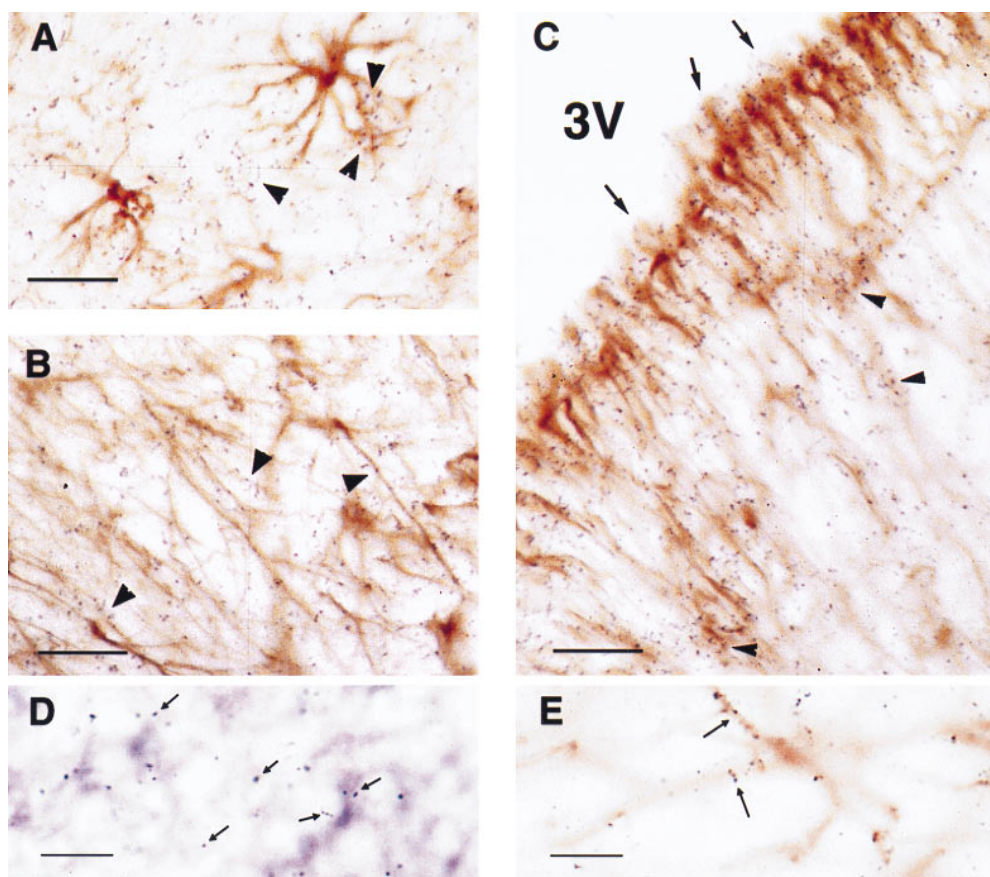


FIG. 4. Localization of D2 mRNA in astrocytes and tanycytes. Tissue sections were immunostained for glial fibrillary acidic protein after *in situ* hybridization with the antisense D2 riboprobe, and the sections were coated with film emulsion and exposed for 20 days. (A) Immunopositive cells (astrocytes) of layer 1 of the cerebral cortex. Some silver grains can be observed in the cell bodies, but the majority are located along the cell processes (arrowheads). (B) Molecular layer of the dentate gyrus. Astrocyte fibers can be seen crossing this region. Hybridization grains (arrowheads) colocalized with the staining. (C) Lower part of the third ventricle (3V). Silver grains are seen on the cell bodies of tanycytes lining the ventricle and on the cellular processes crossing into the adjacent arcuate nucleus. (D) Lack of correlation between D2 mRNA (silver grains, arrows) and neuronal-specific enolase mRNA (blue spots) in the granular cell layer of the cerebellum. (E) Presence of D2 mRNA (silver grains, arrows) in GFAP-stained astrocytes of the granular layer of cerebellum. Photomicrographs were taken with a 40 \times (A–C) or 63 \times (D and E) immersion objective. [Bars = 20 μ m (A–C) and 10 μ m (D and E).]

form contributes significantly to the neuronal pool of T3 in the neonatal brain.

In summary, our observations suggest that astroglial cells play a central role in thyroid hormone homeostasis in the developing central nervous system and may modulate neuronal and oligodendrocyte function through the production and release of T3. This concept raises the intriguing possibility that neuronal processes might influence astroglial D2 activity through local regulatory mechanisms. In this context, the demonstrated effects of adrenergic agents and other substances to stimulate D2 activity in primary cultures of rat neonatal astroglial cells may be of relevance (46–48).

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