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Tyrosine hydroxylase mRNA-containing neurons in the medial amygdaloid nucleus and the reticular nucleus of the thalamus in the Syrian hamster

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To confirm previous immunocytochemical findings in colchicine-treated Syrian hamsters, in situ hybridization was used to investigate the distribution of TH mRNA-containing cells in the medial amygdaloid nucleus (Me) and the thalamic reticular nucleus (Rt) of untreated hamsters. TH mRNA-producing neurons were observed in anterior and posterior Me and throughout Rt, similar to the distribution of TH-immunostained cells in these areas of animals receiving colchicine. These data confirm that TH is normally produced in amygdaloid and thalamic cell groups which lie outside the classical catecholamine systems.

The distribution of catecholamine-containing cells in the central nervous system, as originally described using formaldehyde-induced histofluorescence[5], has been confirmed by the immunocytochemical localization of tyrosine hydroxylase (TH)[17], the rate-limiting enzyme in catecholamine biosynthesis. Recently, however, TH-producing neurons have been reported in numerous brain regions and in a variety of mammalian species that are outside the classically-described catecholaminergic systems[8,10,11,14,16,18,23,24,26,28,29,40,41,44]. One such area is the medial amygdaloid nucleus (Me) of the Syrian hamster (*Mesocricetus auratus*)[1,2,6,42]. This nucleus is a major relay area for incoming olfactory and vomeronasal signals[22,38] and projects to the medial preoptic area and hypothalamus[12]. While studying the amygdaloid TH neurons, Asmus et al.[2] also observed a limited number of TH-immunostained cells in the reticular nucleus of the thalamus (Rt) in the hamster, another region that lies outside of the previously-described catecholaminergic cell groups. The Rt is a thin sheet of GABAergic neurons[7,19] located at the lateral boundary of the dorsal thalamus that receives collateral inputs from traversing thalamocortical and corticothalamic fibers[20].

Although a limited number of TH-immunoreactive neurons are observed in Me[2,6,42] and Rt (unpublished observations) of hamsters not treated with colchicine, the full extent of TH-immunostained cells in these regions are observed only in animals that have received intracerebroventricular injections of colchicine[1,2], an axoplasmic transport inhibitor[4] commonly used to enhance immunostaining of various substances. However, this drug has been shown to induce de novo expression of mRNAs for various neuropeptides[3,25,36]. To determine whether the immunocytochemical findings in the hamster were an artifact of the drug treatment, we examined the distribution of TH mRNA-containing cells in Me and Rt of untreated hamsters using in situ hybridization.

Four adult male Syrian hamsters were anesthetized with sodium (Na) pentobarbital (15 mg/100 g b.wt., i.p.) and perfused transcardially with isotonic saline containing 0.1% Na nitrite, followed by 250 ml of 4% paraformaldehyde in 0.1 M Na phosphate buffer (pH

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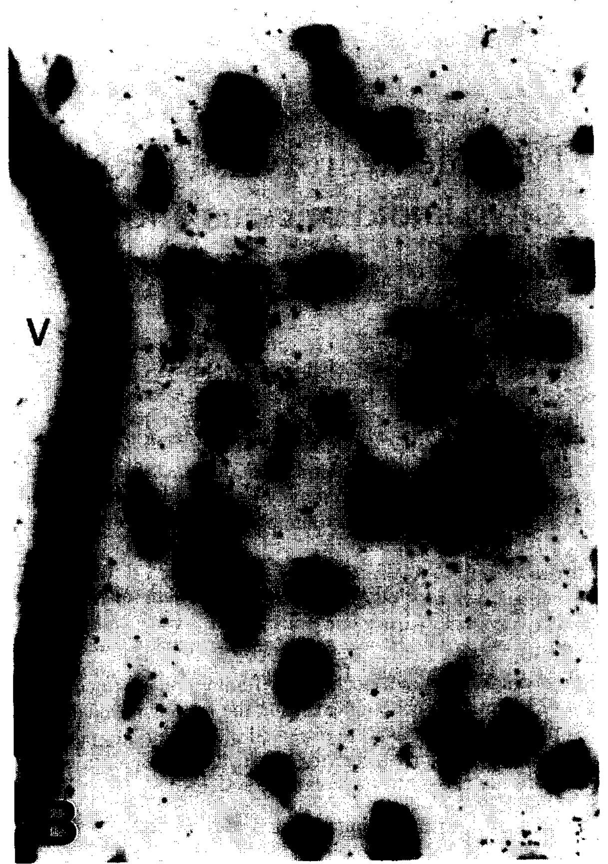
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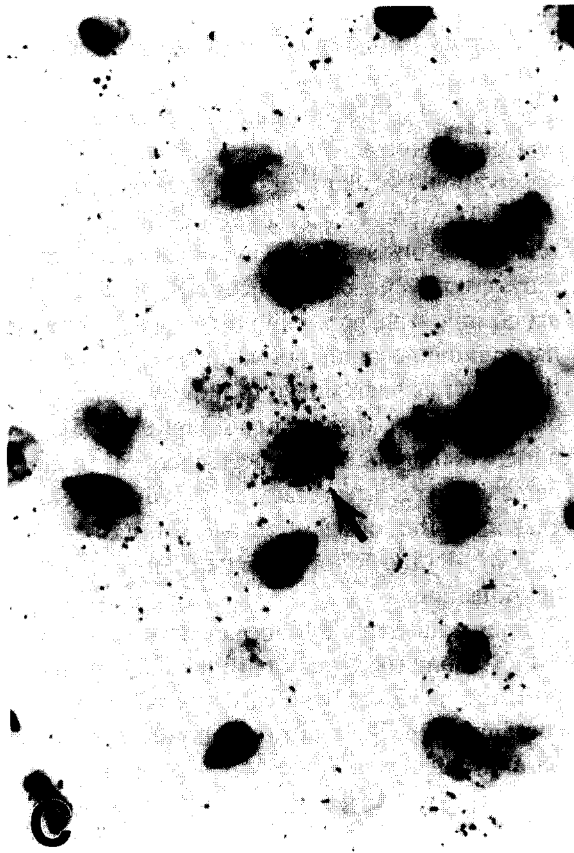
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7.4). Brains were removed and postfixed for 1 h in the perfusion fixative. An additional brain was removed from an anesthetized, non-perfused hamster. All brains were frozen in isopentane cooled with liquid nitrogen and stored at $-80°C$. Coronal brain sections (12 μm) were cut on a cryostat at -18 to $-20°C$, thaw-mounted onto poly-L-lysine-coated slides, and stored at $-80°C$.

On the day of hybridization, sections from the unperfused brain were fixed in 4% paraformaldehyde in 0.1 M PBS for 15 min. All sections were placed in proteinase K (10 μg/ml perfused tissue; 2 μg/ml immersion-fixed tissue) in TE buffer (100 mM Tris, 50 mM EDTA, pH 8.0) for 30 min at 37°C, immersed in 0.0025% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min at room temperature, and then dehydrated in alcohols prior to hybridization.

T3 polymerase was used to transcribe ^{35}S-labeled antisense cRNA probes from a 1.2 kb cDNA insert cloned into the *Eco*RI restriction site of the pBS(+/−) transcription vector (Stratagene). This cDNA clone (RR 1.2, generously provided by Dr. D. Chikaraishi) corresponds to nucleotides 14–1165 of the rat TH gene sequence[15]. The cRNA probe was purified using NENSORB 20 Nucleic Acid Purification Cartridges (NEN/Dupont), and the specific activity was determined ($2.1–2.3 \times 10^5$ cpm/μl). The probe was diluted to an activity of 1.7×10^4 cpm/μl with hybridization buffer containing 50% formamide, 10 mM Tris (pH 8.0), 1 mM EDTA, 300 mM NaCl, 10% dextran sulfate, $1\times$ Denhardt's, and 100 mM dithiothreitol (DTT). This hybridization solution was applied to the sections and covered with a glass coverslip. After a 16 h incubation at 56°C, the slides were washed and coverslips removed in $2\times$ SSC. Sections were then incubated in RNase A (20 μg/ml) for 30 min at 37°C and washed in decreasing concentrations of SSC containing 1 mM DTT at 56°C ($2\times$, $1\times$ for 10 min each; $0.5\times$ for 60 min), after which sections were dehydrated in alcohols.

The slides were dipped in photographic emulsion (Kodak NTB-2), stored at 4°C for 14–16 days, and developed in Kodak D-19 developer. After counterstaining with hematoxylin and eosin, the sections were dehydrated, cleared with xylene and coverslipped with Permount.

Control experiments were conducted to determine the specificity of the TH probe. Some sections were treated as described above but were incubated with RNase A (20 μg/ml) for 30 min at 37°C prior to hybridization with labeled probe to digest tissue RNA. Other sections were processed as described above but incubated with hybridization solution containing 1.7×10^4 cpm/μl of sense-strand (non-complementary) probe synthesized with T7 polymerase to test for specificity of base pairing between TH mRNA and probe rather than binding due to physical properties. Finally, the distribution of TH mRNA-containing cells was compared to the distribution of TH-immunoreactive cells.

Sections through the amygdala were sampled at 24 μm intervals in all five brains. These sections included all but the most rostral levels of the thalamus. Therefore, a few sections through the rostral thalamus and, as positive controls, a number through the substantia nigra were also hybridized. To quantify the number of labeled cells per section in Me, four sections through anterior Me were examined in two of the five brains. Using brightfield illumination, the number of reduced silver grains over individual cells was counted manually at $500\times$ magnification. A cell was considered labeled if the number of grains over its cytoplasm was $3\times$ greater than that over adjacent, cell-sized areas of neuropil or over neurons in other brain regions that do not contain TH mRNA or protein[32]. To compare the number of TH mRNA-labeled cells in Me in this study with the number of TH-immunoreactive neurons observed previously in colchicine-treated hamsters[1], the average number of labeled cells per section was multiplied by 3.33 to correct for differences in tissue thickness (12 μm vs. 40 μm).

During our prior studies[1,2], the brains of 20 colchicine-treated and 3 untreated hamsters were used for TH immunocytochemistry. The protocols for colchicine administration (200 μg), perfusion with 4% paraformaldehyde, and processing of the brains for immunocytochemistry with a monoclonal TH antibody (Incstar) were as previously described[1].

Cells with dense clusters of grains overlying the cytoplasm were observed in the classically-described catecholaminergic cell groups of the midbrain (A9, A10; Fig. 1A) and hypothalamus (A11–A14; Fig. 1B)[5,17]. In addition, the distribution of TH mRNA-containing cells in the hamster brain was identical to the pattern of TH immunostaining. No specific hybridization signal was observed in sections pretreated with RNase or incubated with the sense-strand probe. Based

Fig. 1. Photomicrographs of TH mRNA-containing cells in (A) substantia nigra, (B) paraventricular nucleus of the hypothalamus adjacent to the third ventricle (v), (C) medial amygdaloid nucleus (one labeled cell at arrow), and (D) reticular nucleus of the thalamus (two adjacent labeled cells at arrows). Bar = 20 μm.

on these findings, the TH cRNA probe used in this study is concluded to hybridize specifically with TH mRNA.

In all brains, distinct groups of TH mRNA-containing cells were observed in both the amygdala and the thalamic reticular nucleus. In the amygdala, TH mRNA-producing cells were observed predominantly in the anterior and posterior regions of Me (Fig. 1C), while labeled cells were also scattered in the anterior amygdaloid area, the anterior, ventral portion of the central nucleus of the amygdala and in the intra-amygdaloid bed nucleus of the stria terminalis. This distribution matches that observed with TH immunocytochemistry here and in previous studies[1,2]. The TH mRNA-producing cell groups in anterior and posterior Me were identical in location to the distribution of TH-immunostained cell groups in colchicine-treated hamsters, with the majority of labeled cells concentrated in the dorsal subdivisions of each of these areas. Furthermore, in two of the brains, the number of labeled cells per 12 μm section through anterior Me ranged from 7–14, with a mean of 10.4. To estimate the number of TH-mRNA-containing cells present in a 40 μm section, this mean was multiplied by 3.33 to yield a value of approximately 35 cells per section. Although well above background, the density of grains per labeled cell in Me generally was less than that observed in cells in the substantia nigra and the para- and periventricular hypothalamic nuclei (Fig. 1A,B).

A large number of TH mRNA-containing cells was also observed in Rt throughout its rostrocaudal and dorsoventral extent, with no apparent topographic distribution (Fig. 1D). Labeled cells in the posterior region of Rt were continuous ventromedially with the TH-producing cells of the zona incerta.

As reported previously[1,2,6,42], a limited number of TH-immunoreactive neurons were found in Me of untreated animals, and colchicine treatment dramatically increased this number. In contrast to the TH cells in Me, colchicine treatment only slightly enhanced TH immunolabeling in Rt. In Rt of both treated and untreated brains, the TH-immunostained cells contained a faint light brown reaction product (Fig. 2A), compared to the intense black product seen in TH-immunostained neurons in Me (Fig. 2B) and other brain regions.

The distribution of TH mRNA-containing cells in Me and Rt observed with in situ hybridization in this

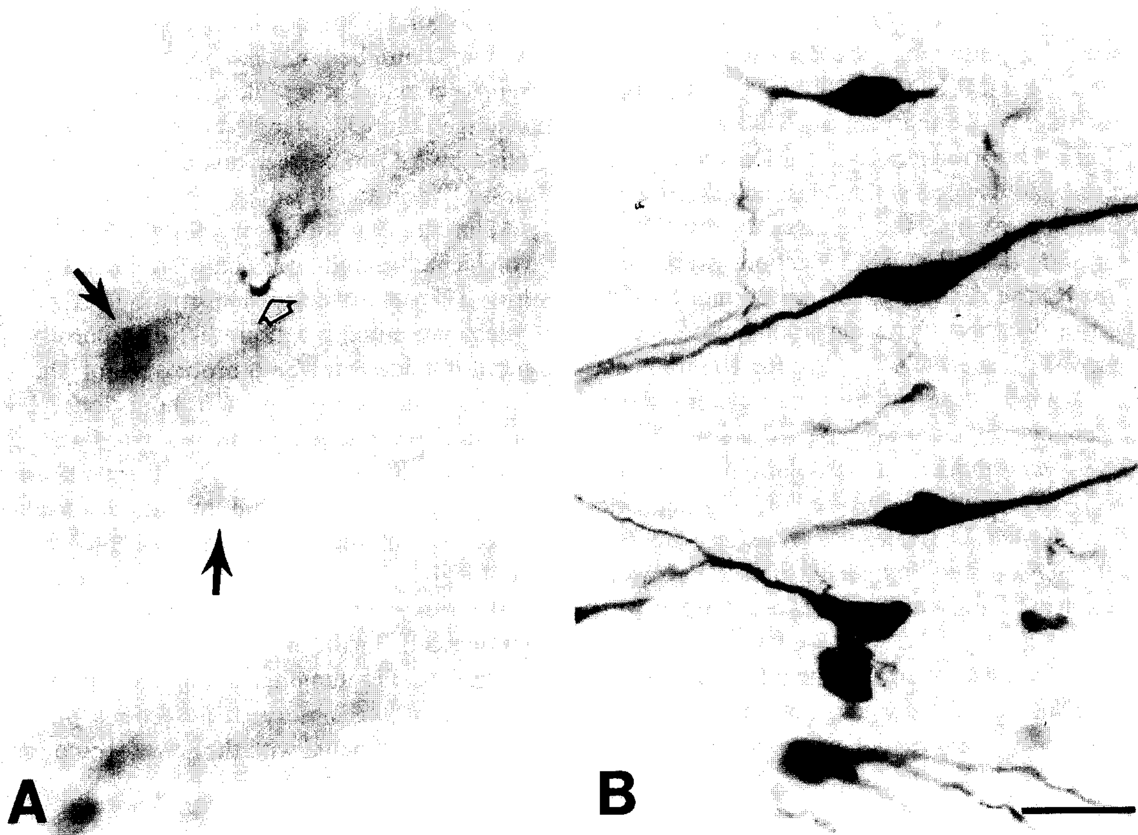

Fig. 2. Photomicrographs of TH-immunoreactive neurons in (A) reticular nucleus of the thalamus and (B) medial amygdaloid nucleus of colchicine-treated hamsters. Note the faint TH immunostaining in cells of the reticular thalamic nucleus (solid arrows) relative to the dark immunolabeling of a nearby TH-immunoreactive fiber (open arrow) and to TH-labeled cells in the medial amygdaloid nucleus (B). Bar = 20 μm.

study was similar to that observed using TH immunocytochemistry in colchicine-treated hamsters[1,2]. Furthermore, after correcting for differences in section thickness, the number of TH mRNA-containing cells was equivalent to the average number of TH-immunostained neurons in sections through Me of animals receiving the drug[1]. These results support our previous assumption that colchicine administration enhances immunostaining by blocking axoplasmic transport rather than by inducing de novo synthesis of TH in the amygdala and thalamus of the hamster.

The observation of TH mRNA-containing cells in the hamster Me and Rt adds to a growing list of TH-producing cells that are located outside of the classically-described catecholaminergic systems, and this distribution is often species-specific[1,2,6,8,10,11,14,16,18,23,24,26,28,29,40–42,44]. The unique pattern of TH expression observed in the Syrian hamster may be due to species differences in the regulatory regions of the TH gene[9].

In many of these novel TH cell groups, the final catecholamine being produced is not known. Previous studies in the hamster have shown that neither the amygdala nor the thalamus contain neurons immunostained for dopamine-β-hydroxylase or phenylethanolamine-*N*-methyltransferase[2,42], enzymes that synthesize norepinephrine and epinephrine, respectively. Dopamine-immunostained neurons were observed in the posterior region of Me, but not in anterior Me[2] or in Rt (unpublished observations), suggesting that the TH-producing cells in posterior Me synthesize dopamine. Whether the TH-containing cells in anterior Me and Rt produce aromatic amino acid decarboxylase (AADC), the enzyme that converts L-DOPA to dopamine, awaits further study. Several reports indicate that some TH-producing cells may not synthesize AADC and that the endproduct of TH cells lacking this enzyme may be L-DOPA[23,31,43], which has been shown to be released in a transmitter-like fashion[13,33].

At present, the functional significance of the species-specific TH mRNA-containing cells reported here is not known. The medial amygdaloid nucleus is a critical area in a neural circuit that processes chemosensory signals and gonadal hormonal information, both of which are essential for mating behavior in the male hamster[37]. Our previous studies indicate that many of the TH-producing neurons in Me may be influenced by gonadal steroids. A majority of TH-immunostained neurons in the posterior subdivision of this nucleus also contained androgen receptors, and in anterior Me, although fewer TH-immunolabeled cells contained androgen receptors, the number of TH-immunoreactive neurons declined after castration[1]. Thus, the function of the TH mRNA-containing cells observed in both parts of Me may be influenced by gonadal steroids, which are essential for both reproductive[35] and aggressive[27] behaviors in the hamster.

The reticular thalamic nucleus receives information concerning a variety of sensory modalities via collateral inputs from thalamocortical and corticothalamic axons, and this nucleus projects to the same thalamic nuclei from which it receives input[20]. Based on this hodological and physiological evidence, Rt is believed to modulate the activity of thalamocortical neurons[39]. In the rat, the majority of neurons in this nucleus are GABAergic[7,19], and the cat Rt contains both GABA and somatostatin[34]. Preliminary results suggest that the hamster Rt also contains mRNA for glutamic acid decarboxylase (GAD) (unpublished observations), the enzyme which synthesizes GABA. The observation of TH mRNA-containing cells in the hamster Rt suggests that TH and GAD may be colocalized in Rt of this species. The coexistence of TH and GAD has been reported in other regions of the rat brain[29]. Thus, catecholamines, as well as GABA, may play a role in the modulation of thalamocortical signaling in the hamster.

However, although numerous cells in Rt were specifically labeled by the TH cRNA probe, TH immunocytochemistry in colchicine-treated as well as untreated hamsters resulted in extremely faint immunolabeling of cells in this nucleus compared to other TH-immunostained cell groups. One explanation for this weak immunostaining is that the enzyme is rapidly transported out of the cell body. This is unlikely considering that these animals were given an axoplasmic transport inhibitor. Another possibility is that the cRNA probe used here is recognizing another mRNA similar in sequence to TH mRNA. This also appears unlikely due to the negative control results and to the observation of TH-immunostained cells in this brain region. In this regard, the hamster does not appear to synthesize multiple isoforms of TH because Western blotting of hamster brain and adrenal revealed only a single band close in size to that of rat TH[43]. A more plausible explanation is that relatively little of the TH mRNA in these cells is translated into protein. The production of TH is known to be regulated at both the transcriptional and translational levels[21,30]. In conclusion, although TH mRNA is abundant in cells of the hamster Rt, they may produce little, if any, functional TH protein. Future studies should examine the synthesis of AADC, as well as the presence of L-DOPA or dopamine, in this brain region.

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