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A species-specific population of tyrosine hydroxylase-immunoreactive neurons in the medial amygdaloid nucleus of the Syrian hamster

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The medial amygdaloid nucleus (Me) is part of a neural pathway that regulates sexual behavior in the male Syrian hamster. To characterize the neurochemical content of neurons in this nucleus, brains from colchicine-treated adult male and female hamsters were immunocytochemically labeled using antibodies that recognize the catecholamine-synthesizing enzymes, tyrosine hydroxylase (TH), dopamine-β-hydroxylase (DBH) and phenylethanolamine-*N*-methyltransferase (PNMT), as well as dopamine. A large population of TH-immunoreactive (TH-IR) neurons was observed throughout Me of male and female hamsters, primarily concentrated in the midrostral and caudal portions of the nucleus. The somata were generally small to medium in size and bipolar. Brains from animals that did not receive colchicine contained a limited number of TH-IR neurons in Me as reported previously. The DBH and PNMT antisera did not label any cells in Me of colchicine-treated animals, and the dopamine antiserum labeled neurons in the same location as the caudal group of TH-IR cells. Therefore, these caudal TH-IR neurons are interpreted to be dopaminergic. The rostral group of TH-IR neurons, on the other hand, may be producing only the immediate precursor of dopamine, L-3,4-dihydroxyphenylalanine (L-DOPA). The TH-synthesizing neurons in Me of the Syrian hamster appear to be a species-specific group of cells located outside of the previously described catecholaminergic cell groups.

INTRODUCTION

The distribution of catecholaminergic neurons in the rat brain has been well-characterized immunocytochemically using antibodies against tyrosine hydroxylase (TH)[21-23], the first and rate-limiting enzyme in the catecholamine biosynthetic pathway[39,49]. TH-immunoreactive (TH-IR) neurons have also been identified outside of the previously reported catecholaminergic cell groups in the telencephalon of a variety of species, including fish[24,53,57], lizard[64], turtle[64], rat[33,34,68], hamster[7,70], cat[26], non-human primate[10,30], and human[15,16]. The male Syrian hamster (*Mesocricetus auratus*) is the only species in which TH-IR neurons have been reported in the medial nucleus of the amygdala (Me)[7,70].

The medial amygdala plays an essential role in the regulation of male hamster sexual behavior[37,38]. As part of the neural pathway that controls reproduction, this nucleus is the first point at which vomeronasal, olfactory, and hormonal information is integrated[9,17,37,38]. Several putative neurotransmitters have been identified immunocytochemically in somata within Me, including substance P[58], cholecystokinin (CCK)[58,61], somatostatin[13,58], vasopressin[3,65], vasoactive intestinal polypeptide[41], and enkephalin[25] in the rat and substance P[51,67], CCK[44] and dynorphin[50,51] in the hamster. In addition, a sparse number of TH-IR neurons have been identified in Me in studies of the distribution of TH-IR neurons in the hamster brain[7,70], indicating that some neurons in this nucleus may also produce catecholamines. In these studies, animals either were not treated with colchicine[70] or were treated with relatively small doses of the drug[7] to inhibit axoplasmic transport[6,35].

The intracerebroventricular (i.c.v.) administration of colchicine is often necessary to visualize peptide-containing cell bodies with immunocytochemistry[20,40] and has been used occasionally to optimize the identification of TH-IR cells[26,27,62]. Since Davis and Macrides[7] and Vincent[70] observed a small number of TH-containing cells in Me of untreated hamsters, we hypothesized that colchicine treatment would enhance TH immunostaining in this area. Therefore, in this study the brains of colchicine-treated male and female hamsters were utilized to examine the distribution, number and morphology of TH-IR neurons in Me. To determine which of the catecholamines was present in these TH-IR cells, colchine-treated tissue was also incubated with antisera generated against dopamine, or against the enzymes dopamine-β-hydroxylase (DBH) or phenylethanolamine-*N*-methyltransferase (PNMT), which synthesize norepinephrine

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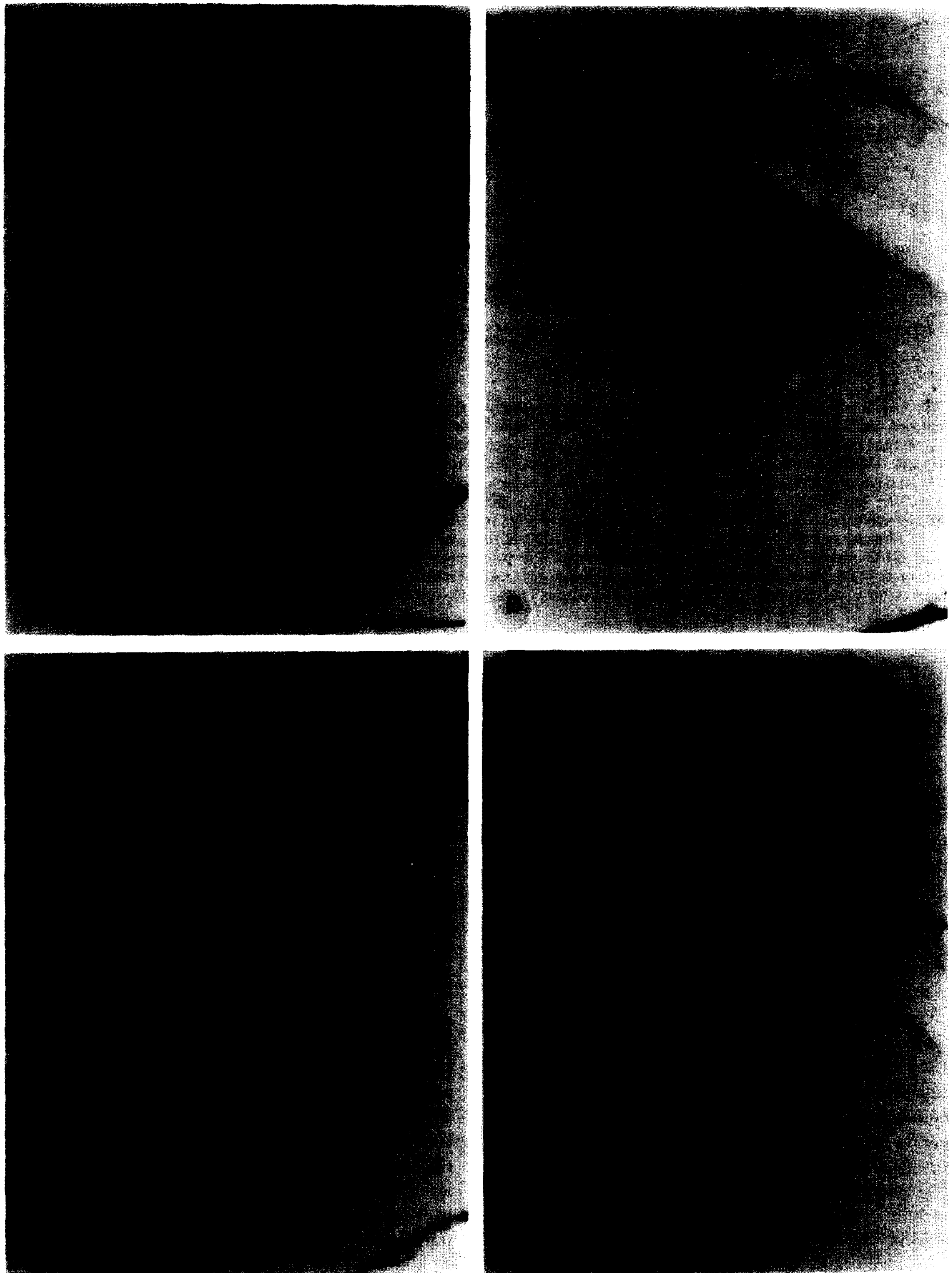

Fig. 1. Photomicrographs of tyrosine hydroxylase-immunoreactive (TH-IR) neurons in the male hamster medial amygdaloid nucleus (Me) immunolabeled with the monoclonal TH antibody. Large populations of TH-IR neurons were observed in midrostral (A) and caudal (B) Me of colchicine-treated brains. Fewer TH-IR neurons were observed in these same areas of noncolchicine-treated brains (C,D). Midrostral Me (A,C) corresponds to the level of Me shown in Fig. 2B, while caudal Me (B,D) corresponds to the level of Me shown in Fig. 2D. In B and D note the close association of TH-IR neurons with blood vessels (B). LV, lateral ventricle; ot, optic tract. Bar = 200 μm.

and epinephrine, respectively.

MATERIALS AND METHODS

Animals, surgery and preparation of tissue

Twenty six adult Syrian hamsters (91–165 g; Charles River, Wilmington, MA) were group-housed in a 14 h light:10 h dark illumination cycle and given food and water ad libitum. To compare dosage effects of colchicine on TH immunostaining, various colchicine concentrations were administered. Thirteen male and 4 female animals were anesthetized with sodium pentobarbital (10 mg/100 g b.wt. i.p.), and were injected stereotaxically into the lateral ventricle with 160 μg (n = 2), 200 μg (n = 13), or 320 μg (n = 2) of colchicine (Sigma, St. Louis, MO) in either 2 or 2.5 μl of distilled H_2O as described by Neal and Newman[50]. Three additional male animals did not receive colchicine. These 20 animals were used for TH, DBH and PNMT immunocytochemistry. Six male animals were used for dopamine immunocytochemistry. Four of these animals received 200 μg of colchicine into the lateral ventricle, and two animals did not receive the drug.

In preparation for TH, DBH, and PNMT immunocytochemistry, hamsters were deeply anesthetized after a survival period of 48 h and perfused through the ascending aorta with 150 ml of 0.1 M sodium phosphate buffered saline (NaPBS) with 0.1% sodium nitrite for vasodilation, followed by 200–250 ml of either 4% paraformaldehyde in 0.1 M sodium phosphate buffer (NaPB) (n = 18, including noncolchicine-treated animals) or 2% paraformaldehyde and 0.25% parabenzoquinone in 50 mM NaPB (n = 2). The benzoquinone-paraformaldehyde fixative was tested because it had been reported to enhance peptide immunostaining in the hamster limbic system[50]. Brains were postfixed for 1–2 h in the perfusion fixative and cryoprotected overnight with 20% sucrose in NaPB at 4°C. Coronal sections (40 μm), cut on a freezing microtome, were serially collected into 0.1 M NaPB with 0.01% sodium azide and stored at 4°C.

Four colchicine-treated and two noncolchicine-treated animals were prepared for dopamine immunostaining by transcardial perfusion with 100 ml of 2% sodium chloride and 1% sodium metabisulfite in dH_2O (pH 7.2) followed by 150 ml of 5% glutaraldehyde and 1% sodium metabisulfite in 50 mM sodium cacodylate buffer (pH 7.6). The brains were removed and blocked into 3–5 mm slices and postfixed for 1 h in the perfusion fixative. The tissue was then transferred to a cryoprotectant solution containing 20% sucrose and 1% sodium metabisulfite in 50 mM Tris (pH 7.2) and stored overnight at 4°C. The cutting and storage procedures were identical to those described above.

Antisera

The polyclonal TH antisera were generated in rabbit against TH purified from either bovine adrenal gland (Eugene Tech Intl., Allendale, NJ) or PC-12 cells (East-Acres Biologicals, Southbridge, MA). The monoclonal TH antibody (Incstar, Stillwater, MN) was raised in mouse against TH purified from PC-12 cells. The DBH and PNMT polyclonal antisera (Eugene Tech Intl.) were raised in rabbit against enzymes purified from bovine adrenal gland. The dopamine antiserum (Eugene Tech Intl.) was raised in rat against dopamine coupled to Limulus hemocyanin by glutaraldehyde. The polyclonal TH, DBH, PNMT and dopamine antisera were used at a dilution of 1:1000, and the monoclonal TH antibody was used at a dilution of 1:7000. Excellent immunostaining was observed when the monoclonal TH antibody was diluted up to 1:50,000.

Immunocytochemistry

For TH, DBH and PNMT immunocytochemistry, free-floating brain sections were immunolabeled using a modification of the Sternberger peroxidase-antiperoxidase (PAP) method[66]. After rinsing in either 0.1 M NaPB or 0.02 M potassium phosphate-buffered saline (KPBS), at least every third section from each brain was incubated with the primary antibody diluted with the same buffer containing 0.3% Triton-X for 48–60 h at 4°C. Sections adjacent to TH-immunostained material from two colchicine-treated brains were incubated with the DBH or PNMT antisera. All subsequent incubations were carried out in KPBS with 0.3% Triton-X for 1 h at room temperature. Depending on the host of the primary antiserum, sections were incubated with either donkey anti-rabbit (1:50) or goat anti-mouse (1:100) antiserum, followed by rabbit (1:100) or mouse (1:200) PAP complex (Jackson ImmunoResearch,

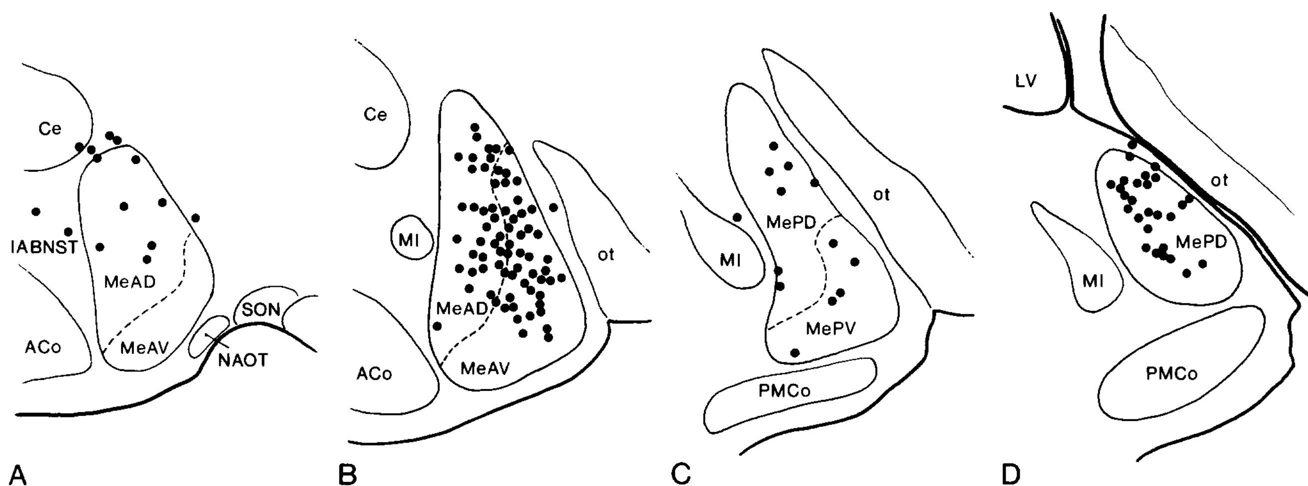

Fig. 2. Schematic representations of coronal sections from 4 rostrocaudal levels of Me from a single hamster brain showing the distribution of TH-IR neurons. A: rostral Me; B: midrostral Me; C: midcaudal Me; D: caudal Me. ACo, anterior cortical amygdaloid nucleus; Ce, central amygdaloid nucleus; IABNST, intraamygdaloid bed nucleus of the stria terminalis; LV, lateral ventricle; MeAD, medial amygdaloid nucleus, anterodorsal subdivision; MeAV, anteroventral subdivision; MePD, posterodorsal subdivision; MePV, posteroventral subdivision; MI, massa intercalata; NAOT, nucleus of the accessory olfactory tract; ot, optic tract; PMCo, posteromedial cortical amygdaloid nucleus; SON, supraoptic nucleus of the hypothalamus.

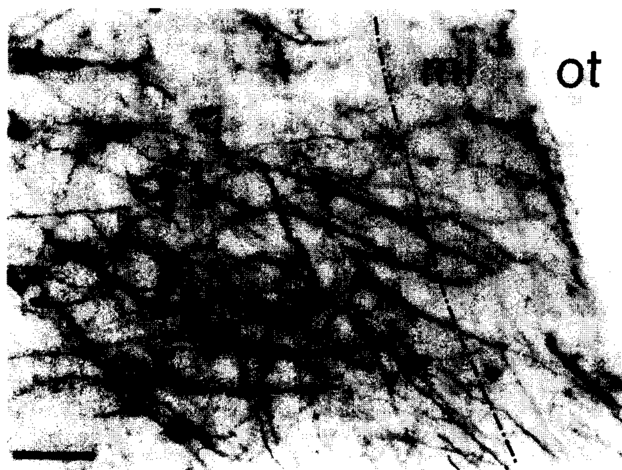

Fig. 3. Photomicrograph of TH-IR neurons in midrostral Me showing the morphological characteristics typical of these neurons throughout the nucleus. Note the dendrites extending into the medial molecular layer (ml) adjacent to the optic tract (ot). Bar = 50 μm.

West Grove, PA). After each incubation, sections were washed 3 times (5 min each) in KPBS. Bound peroxidase was visualized by incubation of the tissue in a solution containing 0.0125% diaminobenzidine tetrahydrochloride (DAB) (Sigma, St. Louis, MO), 0.015% nickel chloride and 0.06% hydrogen peroxide in KPBS for 5–10 min. The reaction was stopped in distilled H_2O, and sections were mounted out of KPBS onto gelatin-coated slides, dehydrated, cleared, and coverslipped with Permount. TH-immunolabeled sections from two brains were counterstained with Cresyl violet.

Dopamine immunostaining was carried out by incubating every other section from the glutaraldehyde-fixed brains with the dopamine antiserum diluted with 1% sodium metabisulfite, 0.5–1% Triton-X, 0.05% bovine serum albumin (BSA) and 0.05% sodium azide in 50 mM Tris (pH 7.2) for at least 12 h at room temperature. The tissue was then incubated for 1 h at room temperature with biotinylated goat anti-rat (Sigma) diluted to 1:100 with 0.1 M NaPBS containing 0.05% BSA, 0.5% Triton-X and 2% normal rabbit serum followed by an incubation with the Elite Vectastain avidin-biotin complex solution (Vector Labs, Burlingame, CA) diluted with the same buffer for 1 h at room temperature. Each incubation was followed by 3 washes in 0.1 M NaPBS containing 0.05% BSA. The bound peroxidase was visualized using the same procedure described previously except that the DAB was dissolved in NaPB. Sections were mounted and coverslipped as described above.

Slides were examined using brightfield illumination on a Leitz Dialux microscope, and TH-immunostained cells were plotted with the aid of a drawing tube. A cell was considered labeled if its soma was filled with dark reaction product and at least one stained process could be seen emanating from the soma. Cell counts reported here were obtained by totaling the number of TH-IR cells in representative sections through midrostral and caudal Me. The diameter of the long axis of TH-IR cells was determined with a measurement reticle.

The specificity of each of the TH antibodies used in this study has been verified previously[24,69,71,75]. Additionally, since purified native TH is not available, blocking studies were carried out by preabsorbing the Eugene Tech TH antiserum with a bacterial lysate containing a TH/β-galactosidase fusion protein (Eugene Tech Intl.) for 4 h at room temperature prior to incubation with the tissue. TH immunostaining was abolished when the primary antiserum was preabsorbed with this fusion protein. No immunostaining was seen when the primary or secondary antiserum was omitted from the respective incubation solution.

RESULTS

TH immunoreactivity

A large population of TH-IR neurons consisting of several hundred cells was observed in Me following colchicine administration. The distribution and morphology of these TH-IR neurons were similar in male and female hamsters. The description that follows is based on our analysis of brains from male hamsters.

The TH-IR neurons were concentrated primarily in the midrostral (Fig. 1A) and caudal (Fig. 1B) regions of this nucleus. In contrast, substantially fewer TH-IR cells were observed in Me of noncolchicine-treated brains (Fig. 1C,D) as reported earlier[7,70]. Both treated and untreated brains contained intensely immunostained neurons in other regions known to contain TH, such as the substantia nigra and locus coeruleus.

A consistent pattern of TH immunolabeling was observed in colchicine-treated brains with all of the TH antibodies used, but the monoclonal and the Eugene Tech polyclonal antibodies provided the best staining qualitatively with our protocol. Brains from animals treated with all 3 doses of colchicine and perfused with either fixative yielded similar results with regard to the distribution, number and morphology of TH-IR cells. Furthermore, the distribution of TH-IR neurons in regions other than Me appeared to be similar to that previously described in noncolchicine-treated hamster brains[70] with the exception that we observed a limited number of immunolabeled cells in the bed nucleus of the stria terminalis and the reticular nucleus of the thalamus.

Analysis of the distribution and number of TH-IR neurons revealed that immunolabeled cells were present throughout the rostro-caudal extent of Me (approximately 1.2 mm) in colchicine-treated brains. A few labeled cells were scattered in the rostral-most portion of Me (Fig. 2A), predominantly in the anterodorsal subdivision of the nucleus as defined by Gomez and Newman[18]. A midrostral group of TH-IR neurons appeared approximately 250 μm from the rostral tip of Me and extended 300 μm caudally (Figs. 1A and 2B). In this region, approximately 50 immunostained cells per 40 μm section were distributed throughout the anterodorsal and anteroventral subdivisions of Me. In the middle and mid-caudal regions of Me, 10 or fewer TH-IR neurons were observed in each section, dispersed throughout the dorsal and ventral subdivisions (Fig. 2C). Another concentrated population of TH-IR neurons was located in the caudal 300 μm of Me where only the posterodorsal subdivision remains (Figs. 1B and 2D). Approximately 30 TH-IR cells were found in single sections through this region of the nucleus.

Although TH immunoreactivity in the amygdala was

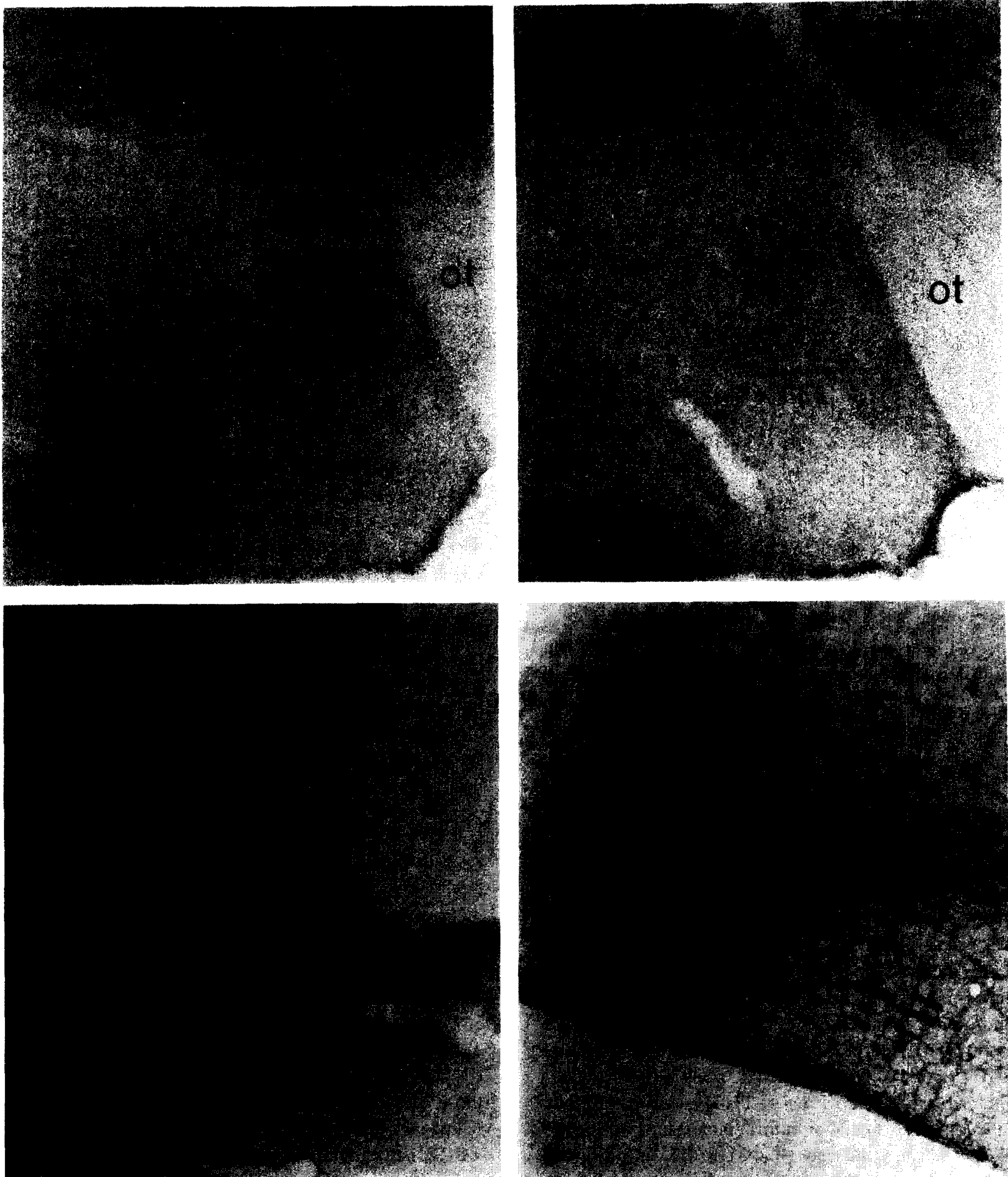

Fig. 4. Photomicrographs A and B show that neither the dopamine-β-hydroxylase (DBH)(A) nor the phenylethanolamine-*N*-methyltransferase (PNMT)(B) antisera labeled any neurons in midrostral Me (level of Fig. 2B). However, neurons of the noradrenergic locus coeruleus from this same brain were intensely labeled with the DBH antiserum (C), and neurons of the adrenergic C1 cell group in the ventrolateral medulla were labeled with the PNMT antiserum (D). ot, optic tract; V, fourth ventricle. Bar = 200 μm.

primarily confined to Me, some immunolabeled cells were also observed in the anterior amygdaloid area, the intra-amygdaloid bed nucleus of the stria terminalis, and the ventromedial region of the central nucleus. The number of immunolabeled neurons in these regions was always less than that observed within Me.

Fig. 5. Photomicrograph of dopamine-IR neurons in caudal Me. The dashed line demarcates the boundary of caudal Me, which consists only of the posterodorsal subdivision. The level of this section corresponds to that of Fig. 2D. MePD, posterodorsal subdivision of Me; ot, optic tract. Bar = 100 μm.

Morphological analysis revealed that the TH-IR somata in midrostral and caudal Me were small to medium in size (diameter of long axis = 8–20 μm) and were mostly fusiform or ovoid (Fig. 3). Pyramidal and spherical perikarya were seen on occasion. The majority of labeled neurons had two primary dendrites emanating from opposite ends of the soma. Cells with 3 primary dendrites were observed less frequently. The morphology of TH-IR neurons in Me was entirely consistent with the description of Golgi-stained neurons in this nucleus[17]. In midrostral Me, processes of TH-IR neurons often extended into the medial or ventral molecular layer of Me, adjacent to the optic tract (Fig. 3) or the ventral surface of the brain, respectively. In the caudal region of Me, TH-IR neurons occasionally surrounded blood vessels (Fig. 1B,D).

DBH, PNMT and dopamine immunoreactivity

No cell bodies in Me were immunostained after incubation of colchicine-treated tissue with either the DBH or PNMT antisera (Fig. 4A,B). However, immunolabeling for these enzymes in other areas of the same brain indicated that the staining procedure was successful (Fig. 4C,D).

When the dopamine antiserum was used to immunolabel the glutaraldehyde-fixed tissue, dopamine-IR neurons were observed in the caudal, but not the rostral, region of Me in colchicine-treated animals (Fig. 5). The location of these dopamine-IR cells corresponded to that of the TH-IR cell group found in the posterodorsal subdivision of Me at the level of Fig. 2D. As many as 22 dopamine-IR neurons were observed in sections through this area. A few dopamine-IR neurons were also present in the posterodorsal region of midcaudal Me at the level of Fig. 2C. No dopamine immunostaining was seen in Me of noncolchicine-treated animals.

DISCUSSION

The large group of TH-IR neurons in the hamster Me revealed by colchicine administration is a novel population of neurochemically-defined cells that have not been described in this nucleus in other species. Although not all of the species studied to date have received colchicine prior to immunocytochemical staining for TH, i.c.v. administration of 100 μg of colchicine into the rat brain[33,34,45] or 200 μg into the cat brain[26] has not revealed TH-IR neurons in Me of either species. In addition, we have not observed TH-IR neurons in the rat Me even after injections of 240 μg of colchicine (unpublished observations), nor was TH mRNA detected in the rat Me using in situ hybridization[29].

This study adds to the growing body of evidence[7,10,15,16,24,26,30,32–34,53,57,64,68,70] for the existence of TH-IR neurons outside of the catecholaminergic cell groups defined in the rat brain by Hökfelt et al.[21–23]. One explanation for the novel expression of TH in selected cell groups in the hamster brain is that species may differ in the DNA elements that facilitate the expression of the TH gene. In support of this hypothesis, Gandelman et al.[14] showed that certain regions of DNA which regulate TH expression are different in rats and humans.

Neither our studies with colchicine-treated brains nor those of Vincent[70] revealed DBH or PNMT immunolabeling in somata of the hamster telencephalon. Thus, the TH-IR neurons in Me apparently do not produce norepinephrine or epinephrine. The dopamine antiserum, however, labeled a subpopulation of neurons in caudal Me in the same location as the caudal group of TH-IR cells, suggesting that these caudal TH-synthesizing cells may be dopaminergic. Although no dopamine immunostaining was observed in midrostral Me, the TH-IR neurons in this area may produce dopamine in lower quantities or with a higher rate of turnover. Alternatively, these neurons may synthesize only the immediate pre-

cursor of dopamine, L-3,4-dihydroxyphenylalanine (L-DOPA). The conversion of L-DOPA to dopamine requires aromatic amino acid decarboxylase, an enzyme which may not be present in all TH-containing neurons[26,43,48,54,63,71].

Although dopamine transmission in a variety of neural systems has been implicated in the sexual arousal of the male rat[1,11,46,55,72] and Me is known to facilitate sexual behavior in the male hamster[37,38], further studies are needed to determine specifically whether the TH-IR cells in Me of the hamster regulate male mating behavior in this species. Elimination of chemosensory inputs to Me[8,47,56,74] or lesions of rostral Me itself[37] completely abolish copulatory behavior in male hamsters. Since the dendrites of TH-IR neurons in midrostal Me extend into the region of the molecular layer containing afferent terminals from the olfactory bulbs (Fig. 3)[37,59], these neurons are in a position to receive behaviorally essential chemosensory information. Alternatively, the TH-IR neurons described in this report may be involved in non-reproductive functions. In the rat, the Me has been shown to influence ingestive[42,52,60] and aggressive[31], as well as sexual[19], behaviors.

The finding that TH-IR neurons in caudal Me were occasionally associated with blood vessels (Fig. 1B,D) is reminiscent of observations of monoaminergic perikarya and dendrites in direct apposition to vascular structures in the primate brain[12]. Felten and Crutcher[12] postulated that these neurons may be influenced by hormones or other blood-borne substances. Neurons in the caudal region of the hamster Me accumulate gonadal hormones[9,36], and their structure is influenced by these steroids. For example, the dendritic morphology of Golgi-stained neurons is altered in caudal Me following castration[17]. The TH-IR neurons associated with blood vessels, therefore, may monitor hormones that influence their morphology and function.

Visualization of the full extent of the TH-containing cell population in Me apparently requires relatively large doses of colchicine since only a few TH-IR cells were detected without the drug treatment, and Davis and Macrides[7] reported that the administration of 10–50 μg of colchicine had no effect on TH immunostaining in Me. It is possible that the administration of colchicine may induce or increase the expression of TH messenger RNA (mRNA) in these cells. Intracerebroventricular injections of colchicine have been reported to alter the mRNA levels for several neuropeptides[4,5,28] and neurotransmitter-synthesizing enzymes, including TH[5], in various regions of the rat brain.

On the other hand, the observation of TH-IR neurons in Me of noncolchicine-treated animals in the present study (Fig. 1C,D), as well as in previous studies[7,70], supports the conclusion that some cells of the hamster Me normally express sufficient quantities of TH to be immunocytochemically detectable. However, immunostainable quantities of this protein may accumulate in the somata of other cells only after the disruption of microtubular transport. This hypothesis is supported by the demonstration that different levels of TH are expressed in separate subpopulations of neurons within the same brain region, as in the substantia nigra[73] and the locus coeruleus[2].

Finally, preliminary studies in our laboratory using in situ hybridization indicate that TH mRNA is expressed in Me of both colchicine- and noncolchicine-treated hamster brains. These preliminary data support the interpretation of the immunocytochemical studies that colchicine simply inhibits the transport of TH in this large, species-specific population of neurons capable of TH expression.

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