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Thyroid Hormone Causes Sexually Distinct Neurochemical and Morphological Alterations in Rat Septal–Diagonal Band Neurons

Anita Westlind-Danielsson, Elizabeth Gould, and Bruce S. McEwen

Laboratory of Neuroendocrinology, Rockefeller University, New York, New York, U.S.A.

Abstract: Sex differences were investigated in cholinergic neurons of the septal–diagonal band region of adult rats subjected to neonatal treatment with 3,3′,5-triiodo-L-thyronine (T_3). Neonatal hyperthyroidism resulted in a 44% increase in specific activity of choline acetyltransferase (ChAT; EC 2.3.1.6) in adult male rat septal–diagonal band region, whereas no change in ChAT activity could be detected in either dorsal or ventral hippocampus. An increase in muscarinic cholinergic receptors, as measured by [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) binding, was discovered in both septum–diagonal band and dorsal hippocampus of the T_3-treated male rats. Immunohistochemistry in the septal–diagonal band region indicated a more intense staining in the neonatally T_3-treated adult male rats than in controls, with larger and more abundant ChAT-positive and nerve growth factor receptor (NGF-R)-positive varicosities. ChAT immunocytochemistry showed a substantial decrease in cell body area in the medial septum and in the vertical limb of the diagonal band of T_3-treated male rats, while cell density increased twofold. Female littermates subjected to the same treatment showed no changes in any of the biochemical or immunohistochemical cholinergic markers. Only in the medial septum was morphology significantly altered in the female T_3-treated rats in that ChAT-positive cell body area increased. These results indicate a marked sexual variation in the septal–diagonal band region with respect to the sensitivity of postnatally developing cholinergic neurons to the actions of excess thyroid hormone. **Key Words:** Thyroid hormone—Choline acetyltransferase—Septohippocampal complex—Sex—Nerve growth factor receptor—Muscarinic receptors. **Westlind-Danielsson A. et al.** Thyroid hormone causes sexually distinct neurochemical and morphological alterations in rat septal–diagonal band neurons. *J. Neurochem.* **56,** 119–128 (1991).

Early experimental hypo- or hyperthyroidism perturbs neuronal organization and is of consequence for mature brain function (Shapiro, 1968; Lipp et al., 1984; Gould and Butcher, 1989; Gould et al., 1990). We sought to determine consequences of neonatal thyroid hormone treatment on the septal population of cholinergic neurons and their fibers. These cells provide the hippocampus with its major cholinergic input (Woolf et al., 1984) and act as pacemakers for the inherent hippocampal theta rhythm (Stumpf, 1965). Because the septohippocampal complex has been implicated in neuroendocrine (Sapolsky et al., 1986), memory, learning (O'Keefe and Nadel, 1978), and neuropathological (Davies and Maloney, 1976) processes, the relevance for studying this brain region is clear.

Relatively little is known about how sex affects development and function of the septohippocampal complex. We recently have reported that morphological features of CA3 pyramidal cells in the rat hippocampus exhibit a similar sensitivity to neonatal thyroid hormone treatment regardless of sex, although the absolute values for these features were sexually dimorphic (Gould et al., 1990). However, fimbrial transection produces a significantly greater axonal sprouting response of sympathetic neurons in the female hippocampus than in the male (Loy and Milner, 1980). Some aspects of cholinergic development in the CNS are observed earlier in females than in males (Loy and Sheldon, 1987). It is likely that sex hormones are direct or indirect determinants of these neuronal reactions. In view of the role of thyroid hormone in brain devel-

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Address correspondence and reprint requests to Dr. A. Westlind-Danielsson at CNS2 Research and Development, ASTRA Research Centre AB, S-151 85 Sodertalje, Sweden.

Abbreviations used: AChE, acetylcholinesterase; acetyl-CoA, acetyl coenzyme A; ChAT, choline acetyltransferase; NGF, nerve growth factor; NGF-R, nerve growth factor receptor; PD, postnatal day; PBS, phosphate buffer; QNB, quinuclidinyl benzilate; T_3, 3,3′,5-triiodo-L-thyronine.

opment, it is interesting that some characteristics of thyroid function differ significantly between the sexes (Christianson et al., 1981; Gayo et al., 1986). Also, higher basal and stimulated levels of thyroid-stimulating hormone in the male are mediated by the actions of testosterone (Christianson et al., 1981). Sexual comparisons of thyroid function and tissue-specific sensitivity to thyroid hormones are of particular clinical interest in view of the fact that marked sexual differences exist in the incidence of virtually all thyroid diseases in humans (Gregerman, 1986).

Choline acetyltransferase (ChAT; EC 2.3.1.6) is a well accepted marker for cholinergic neurons (Fonnum, 1969; Frotscher and Leranth, 1985), and nerve growth factor receptor (NGF-R) colocalizes with these neurons to a high degree (Hefti et al., 1986; Woolf et al., 1989). We have combined biochemistry (ChAT enzyme activity) and immunohistochemistry (ChAT immunoreactivity and NGF-R immunoreactivity) to explore whether sex is an important determinant in the response of septohippocampal complex development to early excess thyroid hormone treatment.

MATERIALS AND METHODS

Materials

[^{3}H]Acetyl coenzyme A ([^{3}H]acetyl-CoA; 200 mCi/mmol; ChAT assay grade) and [^{3}H]quinuclidinyl benzilate ([^{3}H]-QNB; 43.9 Ci/mmol) were purchased from New England Nuclear Research Products (Boston, MA, U.S.A.). 3,3′,5-Triiodo-L-thyronine (T_3), acetyl-CoA (95% pure), eserine sulfate, choline iodide, and atropine methyl bromide were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Immunohistochemical chemicals were purchased from Vector Laboratories (Burlingame, CA. U.S.A.). Monoclonal antibodies against ChAT were purchased from Boehringer Mannheim (Mannheim, F.R.G.). All other chemicals were of analytical grade.

Animals and thyroid hormone treatment

Thyroid hormone treatment was carried out according to a previously published protocol (Gould et al., 1990). The administered dosages of T_3 [given subcutaneously at postnatal day (PD) 1, 2, and 4] were either 1 μg/g of body weight in 50 μl of sesame oil or 0.5 μg/g of body weight in 25 μl of sesame oil.

Tissue preparation for biochemical analyses

Rats, 23, 64, or 84 days of age, were decapitated at 8–9 a.m. Female rats were used without regard for estrous cyclicity. Dorsal and ventral hippocampus and the septal–diagonal band region were rapidly dissected and immediately frozen on dry ice. A coronal section (approximately 1.8 mm thick) was cut for the dissection of the septal–diagonal band region (see Fig. 1 for the details of this dissection). Tissue was stored at −80°C 1 week or less prior to assay.

Tissue was homogenized in 10 volumes of 10 m*M* Tris-base (pH 7.4 at 4°C), 1 m*M* EDTA, 20 m*M* molybdate, 10% glycerol, and 1 m*M* dithiothreitol with 10 strokes and 2,000 rpm in a glass–Teflon homogenizer at 4°C. Aliquots of homogenate were diluted 1:2 with Triton X-100 and EDTA to give a final concentration of 0.5% (vol/vol) and 10 m*M*, respectively, and ChAT activity was assayed immediately. Tissue from two rats was pooled when determining ChAT activity from the septum–diagonal band.

After another 10 strokes, the homogenate was centrifuged at 68,000 *g* in a Beckman 50Ti rotor and a L3-50 ultracentrifuge (Beckman, Fullerton, CA, U.S.A.) for 30 min at 4°C. The resulting crude membrane pellet used for the measurement of muscarinic cholinergic receptors was either processed immediately (for experiment depicted in Table 2) or frozen on dry ice and stored at −80°C (for experiment depicted in Fig. 3).

ChAT enzyme assay

ChAT activity was determined principally according to Fonnum (1969) as modified by Casper and Davies (1988). All samples were assayed in quadruplicate. Values for the nonspecific activity, assessed at an incubation temperature of 0°C, were subtracted from the total activity.

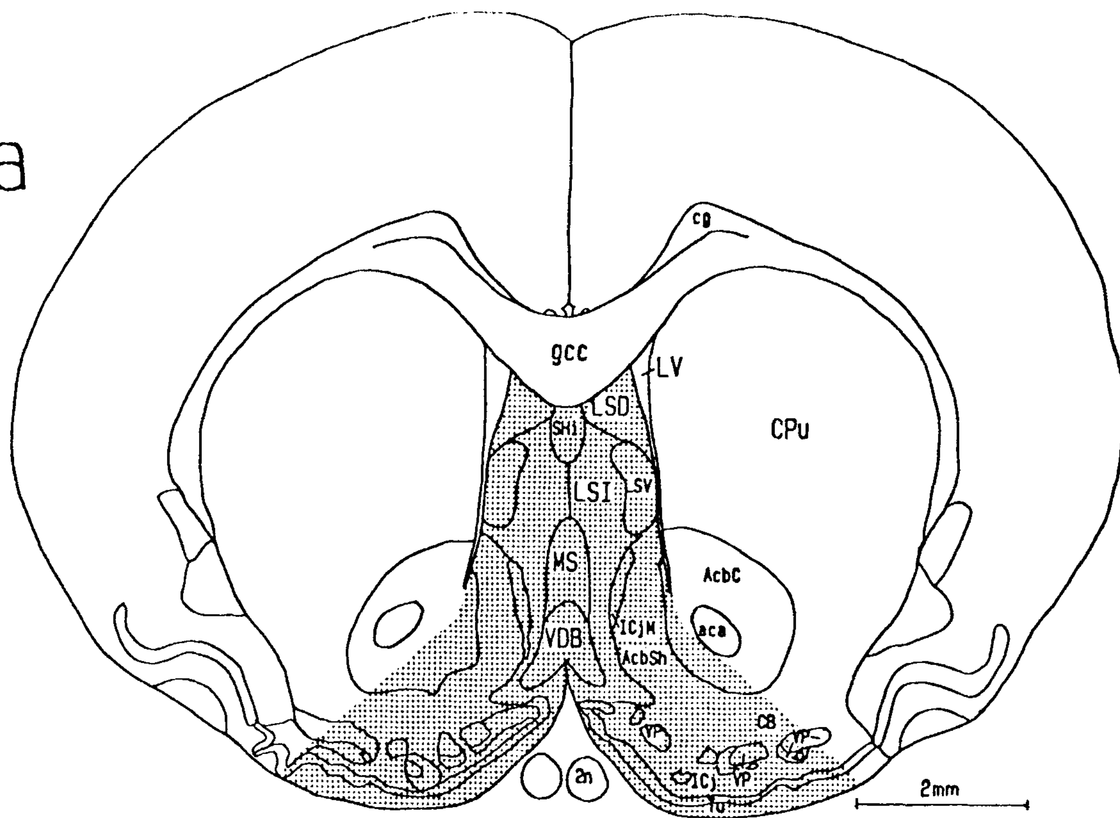
Random sampling showed that ChAT enzyme activity, in the presence of various concentrations of choline (0.05–1.0 m*M*), followed simple Michaelis–Menten kinetics and showed a K_m value of 0.187 ± 0.004 m*M* (mean ± SEM, n = 6) for choline when analyzed with an Eadie–Hofstee plot.

Enzyme activity was found to be linear with time and protein concentration well within the limits used in this study. Enzyme activity is expressed as nanomoles of [^{3}H]acetylcholine formed per hour and milligram of protein (nmol/h/mg of protein).

Muscarinic cholinergic receptor equilibrium binding assay

Frozen crude membrane pellets were thawed on ice and homogenized at 4°C in a buffer (A) of the following composition: 5 m*M* HEPES (pH 7.4), 137 m*M* NaCl, 2.68 m*M* KCl, 1.05 m*M* $MgCl_2$, 1.8 m*M* $CaCl_2$, and 1 g/L glucose. Approximately 70–150 μg of protein was incubated in 1 ml of buffer A with 5 n*M* [^{3}H]QNB. The samples were carried out in triplicate. Nonspecific binding was measured in the presence of atropine (10 μM). Typically, nonspecific binding was about 25% of the total binding. Samples were incubated at room temperature for 120 min and bound ligand was sep- →

FIG. 1. Approximate rostral (**a**) and caudal (**b**) planes (+1 and −0.8 mm from bregma, respectively) between which the septum–diagonal band was dissected for use in the biochemical assays (see shaded region, a). a: 2n, optic nerve; aca, anterior portion of anterior commissure; AcbC, core of accumbens nucleus; AcbSh, shell of accumbens nucleus; CB, cell bridges of ventral striatum; cg, cingulum; CPu, caudate-putamen; gcc, genu corpus callosum; ICj, islands of Calleja; ICjM, islands of Calleja, major island; LSD, dorsal portion of lateral septal nucleus; LSI, intermediate portion of lateral septal nucleus; LSV, ventral portion of lateral septal nucleus; LV, lateral ventricle; MS, medial septal nucleus; SHi, septohippocampal nucleus; Tu, olfactory tubercle; VDB, nucleus vertical limb of the diagonal band; VP, ventral pallidum. b: 3V, third ventricle; AA, anterior amygdaloid area; AC, anterior commissural nucleus; B, basal nucleus of Meynert; BAC, bed nucleus of the anterior commissure; BSTLP, bed nucleus of the striatal terminal, lateral, posterior; BSTMPM, bed nucleus of the striatal terminus, medial, posteromedial; cc, corpus callosum; df, dorsal fornix; f, fornix; GP, globus pallidus; HDB, nucleus of the horizontal limb of the diagonal band; LPO, lateral preoptic area; MCPO, magnocellular preoptic nucleus; MPA, medial preoptic area; MPO, medial preoptic nucleus; MPOC, central portion of medial preoptic nucleus; ox, optic chiasm; Pe, periventricular hypothalamic nucleus; SFi, septofimbrial nucleus; SFO, subfornical organ; SI, substantia innominata; sm, stria medullaris thalamus; SO, supraoptic nucleus; StHy, striohypothalamic nucleus; TS, triangular septal nucleus; vhc, ventral hippocampal commissure. Modified from Paxinos and Watson (1986).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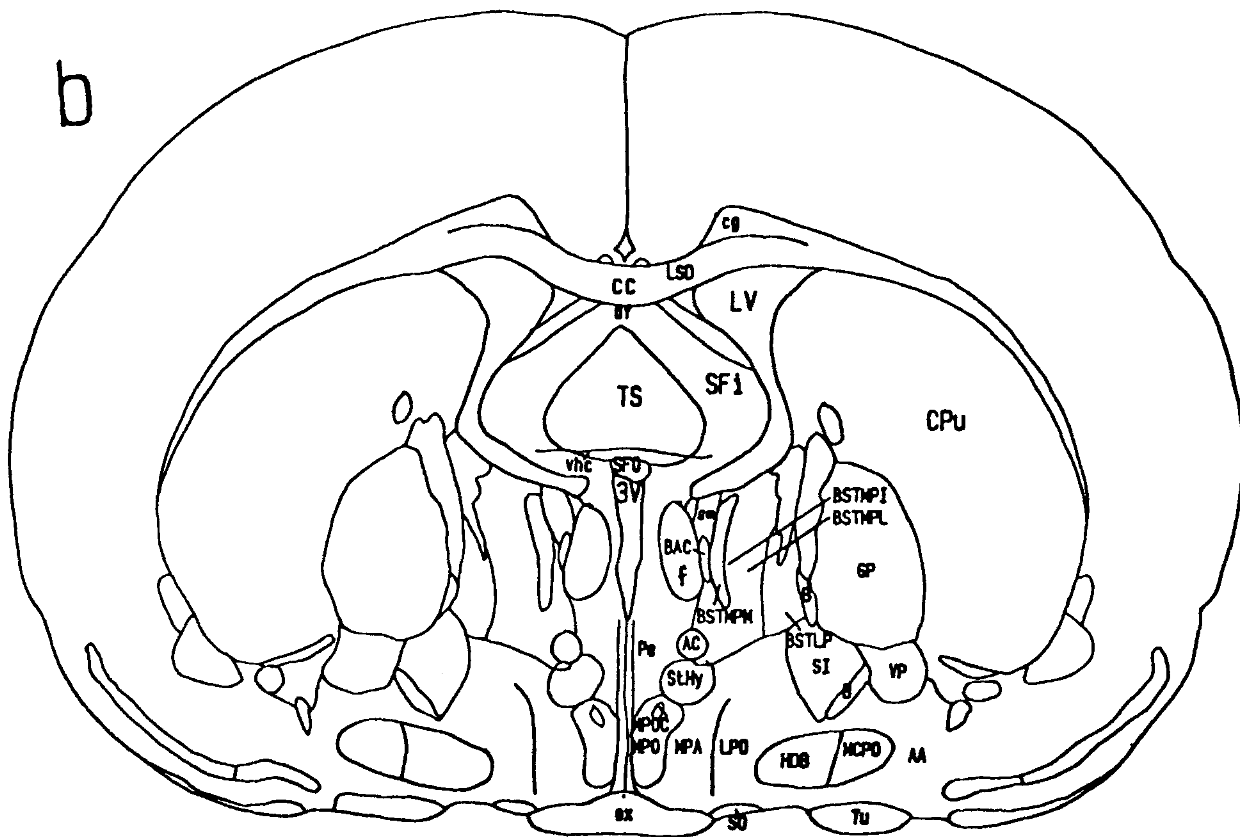
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arated from free ligand by filtration technique on Whatman GF/C filters. Filters were then washed twice with 2 × 5 ml of ice-cold buffer and subsequently counted in a liquid scintillation counter in a 6-ml cocktail of Ready Safe scintillation fluid (Beckman). Neither the ChAT activity nor the [^{3}H]QNB receptor binding assay was affected by the components in the Tris-EDTA-molybdate-glycerol-dithiothreitol buffer as compared with tissue homogenized in water alone.

Protein

Protein measurements were assayed according to the method of Peterson (1977) using the Folin–Ciocalteu's phenol reagent. All samples were assayed in quadruplicate using bovine serum albumin as a standard.

ChAT and NGF-R immunohistochemistry

Immunohistochemistry for ChAT and NGF-R was performed according to previously published protocols (Gould et al., 1989; Woolf et al., 1989). Briefly, 64-day-old animals of both sexes were transcardially perfused with 4.0% paraformaldehyde in 0.1 *M* phosphate buffer (PBS, pH 7.2). Brains were dissected from the cranial cavities and postfixed for 12 h. Sections (50 μm thick) were cut by a vibratome into PBS. The tissue was then incubated in PBS containing either monoclonal antibody to ChAT (Boehringer Mannheim) or monoclonal antibody against NGF-R (code 192; for characterization see Yan and Johnson, 1987) for 24 h. Following several rinses in PBS, the sections were incubated with biotinylated secondary antibody (1:200 in PBS) for 1 h, rinsed in PBS, and placed in avidin-biotin-horseradish peroxidase (1:200 in PBS) for 1 h. Sections were rinsed and reacted in diaminobenzidine with hydrogen peroxide in PBS for 15 min. Nickel ammonium sulfate was added to this last incubation to enhance the reaction product. All brain sections were simultaneously incubated and received identical immunohistochemical treatment. As an immunochemical control, sections from all brains were processed as described above, with omission of the monoclonal antibody incubation.

Analysis of immunoreactive tissue

All slides were coded prior to analysis and the code was not broken until the analysis was completed. At least 40 cells from each brain region were analyzed per animal. Randomly selected ChAT-positive neurons from the vertical and horizontal limbs of the diagonal band and the medial septum were examined for cell body area using an image analysis morphometry program (Southern Micro Instruments). Septal cells were selected from the midline of the medial septum as these neurons appear to represent a morphologically homogeneous population (Swanson and Cowan, 1979). The density of ChAT-immunoreactive neurons in each of the above cholinergic nuclei was determined by counting the number of stained neurons within a 1,000-μm^2 circle placed in the center of each cluster of ChAT-positive cells by use of a camera lucida drawing tube.

Seven coronal sections through the septal region of each brain were examined to determine whether the dimensions of the septum were altered. The septum was traced by use of a camera lucida drawing tube and the cross-sectional areas of these regions were determined using the image analysis morphometry program.

Qualitative observations of the staining intensity of both ChAT-positive and NGF-R-positive cell bodies and processes were made in each of the treatment groups. In addition, the morphological characteristics and staining patterns of immunoreactive fibers, located in putative trajectories and terminal regions of these cholinergic nuclei, were analyzed.

Statistical analysis

Biochemical data (using the absolute values) were analyzed statistically with unpaired two-tailed Student's *t* tests, whereas immunohistochemical data were analyzed with a two-way analysis of variance (treatment × sex) with Tukey HSD post hoc comparisons (Hays, 1981).

RESULTS

Thyroid hormone treatment

The dosages of T_3 were comparable to other early thyroid hormone treatment regimes used to perturb brain development (Gould and Butcher, 1989; Gould et al., 1990). T_3-treated rats used in these experiments were indistinguishable from control rats in appearance. Classical signs of neonatal hyperthyroidism were noted in the neonatal male and female rats subjected to three postnatal T_3 injections: eyes opened 2–3 days earlier (about PD 11) and locomotor activity increased, in line with previously published results (Shapiro, 1968). Both adult body weight and adult adrenal gland weight were significantly lower in the T_3-treated groups compared with controls (Table 1), corroborating earlier findings (Evans et al., 1964; Bakke et al., 1975). The adrenal to body weight ratio was significantly different only between T_3-treated females and same-sex controls.

Specific ChAT activity

Males. Neonatal T_3 injections (1 μg/g of body weight once daily, s.c.) on PD 1, PD 2, and PD 4 produced a

TABLE 1. *Body and adrenal weights from male and female rats, control and neonatally treated with T_3*

	Male		Female	
	Control	T_3 treatment	Control	T_3 treatment
Body weight (g)	308 ± 8.2	272 ± 3.8^b	225 ± 5.4	208 ± 3.8^a
Adrenal weight (mg)	41.8 ± 1.7	35.0 ± 1.9^a	75.0 ± 3.2	57.7 ± 2.6^c
Adrenal/body weight (10^{-6})	135 ± 3.2	129 ± 3.5^d	340 ± 10	272 ± 10^c

The above weights (mean ± SEM) were recorded for male and female rats, 64 and 84 days of age, respectively, that were neonatally treated with T_3 (1 μg/g of body weight given once daily s.c.) on PD 1, 2, and 4, or given the vehicle (controls). Each value is the result of an n = 8–10.

$^a p < 0.05$, $^b p < 0.01$, $^c p < 0.001$, ^{d}not significant versus the same sex controls (Student's two-tailed *t* test).

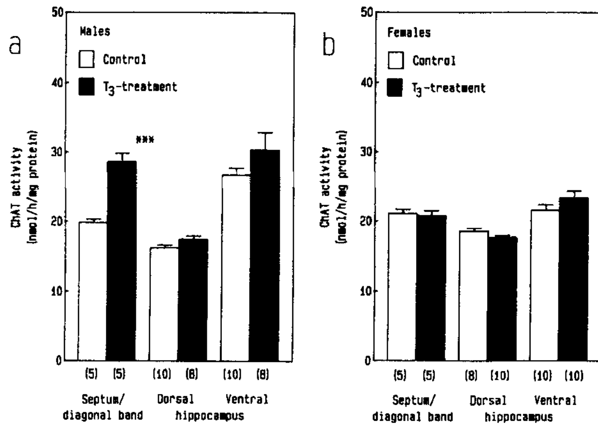

FIG. 2. ChAT activity (nanomoles per hour per milligram of protein) as a function of brain region in male (**a**) and female (**b**) control and neonatally T_3-treated (1 μg/g of body weight, s.c.) rats. Numbers in parentheses under the bars indicate the number of rats used for each experiment (mean ± SEM). ***$p < 0.001$.

marked increase (44.4%; $p < 0.001$) in the specific activity of ChAT in homogenates from the septum–diagonal band of 64-day-old male rats (Fig. 2a). No significant change in enzyme activity was detected in either dorsal or ventral hippocampus when controls were compared with T_3-treated rats (Fig. 2a). ChAT activity in ventral hippocampus, however, did exceed that of dorsal hippocampus (Fig. 2a), as has been reported earlier (Bakke et al., 1975).

A lower dosage of T_3 (0.5 μg/g of body weight, s.c.) resulted in similar increases in ChAT activity in the septum (45%; $p < 0.05$) and in the diagonal band region (35%; $p < 0.05$), analyzed separately in this experiment (Fig. 3a). Again, no change was noted in the dorsal or ventral hippocampus (data not shown). Similar to earlier findings (Hoover et al., 1978), ChAT activity in the diagonal band was significantly higher (2.2-fold) than in the septum (Fig. 3a). This difference was apparent regardless of treatment.

Females. In sharp contrast to the data in males, ChAT activity in T_3-treated female rat septal–diagonal band region showed no tendency to differ significantly from values in the control females (Fig. 2b). Similar to the data in males, no increase in ChAT activity was seen in the female dorsal or ventral hippocampus following T_3 treatment (Fig. 2b). For practical purposes, results depicted in Fig. 2b are from 84-day-old females, whereas males were 64 days of age. However, separate experiments showed that results from 64- and 23-day-old females did not differ from results in 84-day-old females: ChAT activity in 23-day-old T_3-treated females was 108.2 ± 5.0% of control (N = 4) and in 64-day-old T_3-treated females, it was 95.2 ± 10.7% of control (N = 10).

Equilibrium binding studies of muscarinic receptors

Males. Muscarinic cholinergic receptors were measured with saturating concentrations (5 n*M*) of the specific high-affinity cholinergic antagonist [^{3}H]QNB (Yamamura and Snyder, 1974). [^{3}H]QNB does not discriminate between the different muscarinic receptor types. Elevation of the B_{max} value for [^{3}H]QNB binding was apparent in a crude particulate fraction from both the septum–diagonal band (21%; $p < 0.05$) and the dorsal hippocampus (17%; $p < 0.05$) of the T_3-treated male rats (Table 2).

When the septum and the diagonal band were analyzed separately in another experiment, both regions exhibited an increase in [^{3}H]QNB binding sites following T_3 treatment, although the elevation was significant only in the septum ($p < 0.05$, Fig. 3b). Muscarinic receptor numbers in control rats were higher in the diagonal band than in the septum (40.6%; $p < 0.01$). This finding is in line with earlier studies (Hoover et al., 1978) and parallels the region-specific difference in ChAT activity depicted in Fig. 3a.

Females. Muscarinic receptor levels in the septum–diagonal band and in the dorsal hippocampus of 84-day-old females did not change following neonatal T_3 treatment (Table 2).

Morphometry of immunocytochemically identified neurons

Light microscopic examination of control sections revealed no nonspecific staining of the secondary antibody throughout the septal–diagonal band regions of all brains.

Effects of T_3 on ChAT immunohistochemistry. Quantitative analysis of ChAT-immunoreactive tissue revealed significant differences in cell body area in both the medial septum and vertical limb of the diagonal band across treatment groups [$F(3,8) = 19.2$; $p < 0.005$ for medial septum; $F(3,8) = 7.3$; $p < 0.025$ for vertical limb of the diagonal band, see Table 3]. Significant differences in cell body area also were observed between sexes; cell body area was larger in the control male

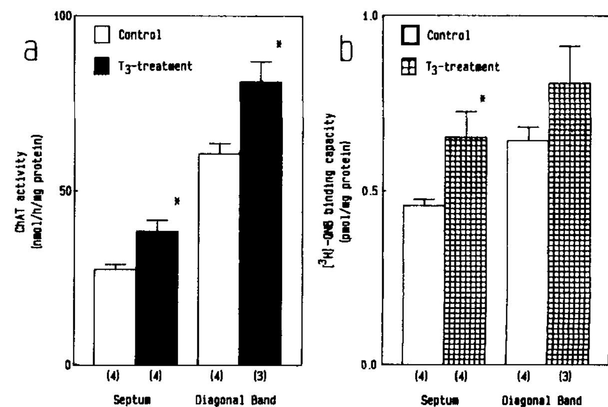

FIG. 3. a: ChAT activity (nanomoles per hour per milligram of protein) determined in homogenates from septum and diagonal band of 84-day-old male control rats or T_3-treated rats (0.5 μg/g of body weight, s.c.). Numbers in parentheses indicate the number of rats used for each experiment (mean ± SEM). **b:** Muscarinic cholinergic binding capacity (picomoles per milligram of protein) measured with [^{3}H]QNB (5 n*M*) in the same groups of animals and brain regions represented in a. *$p < 0.05$.

TABLE 2. *Muscarinic receptors as measured by [^{3}H]QNB binding capacity in the septum/diagonal band and dorsal hippocampus in male and female rats following neonatal treatment with T_3*

	Male				Female			
Brain region	Control	(n)	T_3 treatment	(n)	Control	(n)	T_3 treatment	(n)
Septum/diagonal band	0.81 ± 0.03	(5)	0.986 ± 0.04[a]	(5)	0.58 ± 0.01	(5)	0.59 ± 0.01[b]	(5)
Dorsal hippocampus	1.26 ± 0.09	(7)	1.476 ± 0.03[a]	(10)	0.78 ± 0.03	(10)	0.77 ± 0.03[b]	(9)

B_{max} values (mean ± SEM, in pmol/mg protein) for equilibrium binding of [^{3}H]QNB are given for male (64-day-old) and female (84-day-old) rats which were neonatally treated with T_3 (1 μg/body weight/day) on PD 1, 2, and 4 or given the vehicle (control). Details for the measurement of muscarinic receptors are given in Materials and Methods. The n ranged from 5–10 as indicated above.
[a] $p < 0.05$, [b] not significant versus the same sex controls (Student's two-tailed t test).

than in the control female ($p < 0.05$, Table 3). Differences were noted as well between the control and T_3-treated animals. It is noteworthy that the nature of these differences appeared to depend on sex; T_3 treatment resulted in larger cell body areas in females, whereas smaller cell body areas were observed in the T_3-treated males ($p < 0.05$ for both comparisons, Table 3). No significant differences in cell body area were detected in the horizontal limb of the diagonal band [$F(3,8) = 1.1$; $p > 0.25$].

Quantitative analysis of the density of ChAT-positive neurons revealed significant sex and T_3 treatment differences in both the medial septum and the vertical limb of the diagonal band [$F(3,8) = 10.1$; $p < 0.005$ for the vertical limb]. A sex difference in the density of these neurons was observed in the control but not in the T_3-treated animals; females demonstrated more densely packed neurons in the medial septum and vertical limb of the diagonal band than did males ($p < 0.05$, Table 3). In addition, T_3 treatment appeared to result in a significantly greater density of cells in the male medial septum than in the same-sex controls ($p < 0.05$); no T_3 effect was evident in females. No significant differences were observed in the density of ChAT-positive cells in the horizontal limb of the diagonal band ($p < 0.25$ for all comparisons, Table 3).

In addition to the apparent changes in the size and density of ChAT-positive cells in the medial septum, the cross-sectional area of the septal region was substantially altered with neonatal T_3 treatment [$F(3,8) = 17.1$; $p < 0.005$). In males, T_3 treatment resulted in a significantly smaller cross-sectional area of the septum compared with controls (0.467 ± 0.039 versus 0.654 ± 0.017 mm^2; $p < 0.01$). In contrast, T_3 treatment of females resulted in a significantly larger cross-sectional area of the septum compared with controls (0.653 ± 0.014 versus 0.502 ± 0.015 mm^2; $p < 0.005$). A significant sex difference was observed in controls as well (males, 0.654 ± 0.017 mm^2; females, 0.502 ± 0.015 mm^2; $p < 0.005$), which appeared to be reversed with T_3 treatment.

Quantitative analysis revealed a small but significant difference in the number of ChAT-immunoreactive primary dendrites in the medial septum of the T_3-treated female rats [$F(3,8) = 5.4$; $p < 0.05$, Table 3]. No sex difference in the number of primary dendrites was apparent in the untreated rats, nor were significant changes observed in the vertical or horizontal limbs of the diagonal band following T_3 treatment ($p < 0.25$ for all comparisons).

Light microscopic examination of ChAT-positive cell bodies as well as fiber tracts further revealed that

TABLE 3. *Morphological variables measured in the medial septum (ms) and in the ventral (vdb) and horizontal (hdb) limb of the diagonal band of male and female control and T_3-treated rats*

		Male		Female	
Morphological variable		Control	T_3 treatment	Control	T_3 treatment
Cross-sectional cell body area	ms	233.1 ± 6.3	162.4 ± 4.5[a]	186.7 ± 15.3[b]	248.5 ± 6.3[a]
(μm^2)	vdb	244.2 ± 11.9	199.1 ± 7.3[a]	213.6 ± 6.2[b]	238.6 ± 3.4[a]
	hdb	268.4 ± 36.3	221.4 ± 13.5	230.0 ± 8.6	237.4 ± 6.9
Cell density (number of cells	ms	10.3 ± 0.2	19.9 ± 0.4[a]	19.8 ± 2.1[b]	17.4 ± 1.9
per 1,000 μm^2)	vdb	12.7 ± 1.4	24.5 ± 1.5[a]	24.7 ± 1.2[b]	23.1 ± 2.3
	hdb	24.7 ± 1.6	28.9 ± 2.8	26.2 ± 2.2	28.9 ± 2.8
Number of primary dendrites	ms	2.5 ± 0.1	2.4 ± 0.1	2.6 ± 0.1	3.1 ± 0.2[a]
	vdb	3.3 ± 0.2	3.3 ± 0.1	3.7 ± 0.4	3.6 ± 0.1
	hdb	4.4 ± 0.2	4.0 ± 0.1	4.2 ± 0.3	4.3 ± 0.3

Results represent mean ± SEM for each morphological variable obtained as described in Materials and Methods.
[a] $p < 0.05$, compared with same sex control.
[b] $p < 0.05$, compared with opposite sex control.

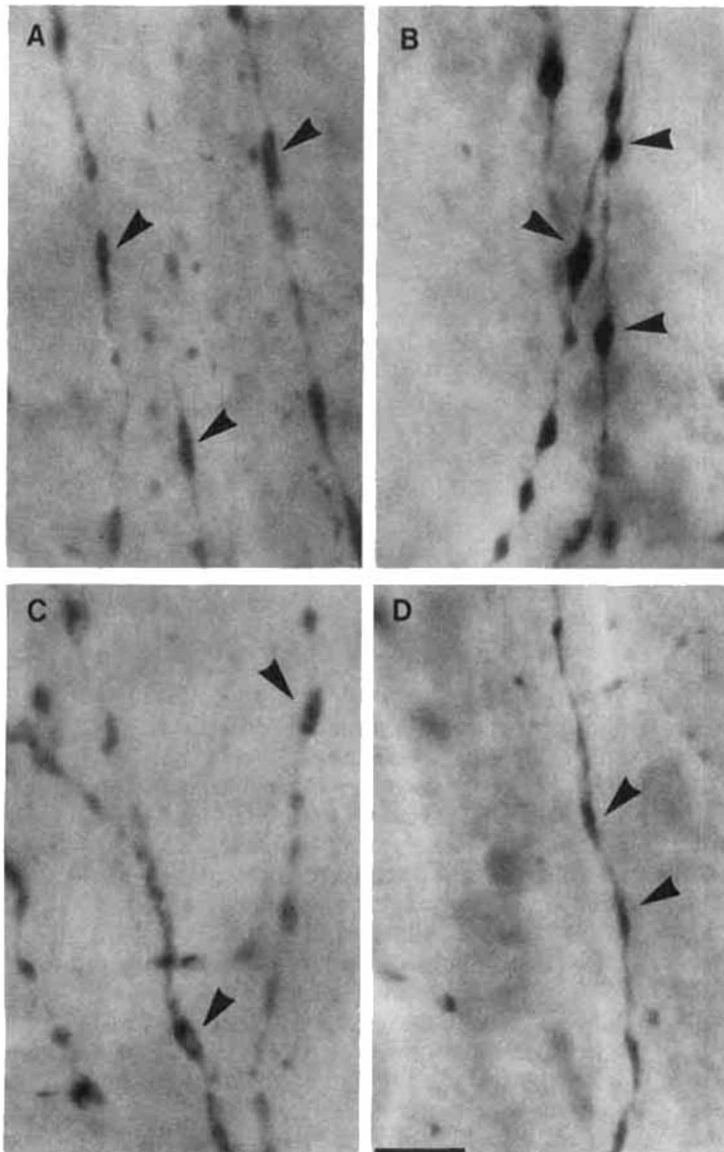

FIG. 4. Representative photomicrographs of ChAT-positive fibers in the medial septum of control (**A, C**) and T_3-treated (**B, D**) male (A, B) and female (C, D) rats. Observe the increase in immunoreactivity and size of varicosities in B compared with A. Note also the lack of difference in staining pattern and morphology of fibers in C compared with D. Arrowheads indicate varicosities. Scale bar in D equals 10 μm and applies to all frames.

T_3-treated males showed more intense immunoreactivity than male controls in both the medial septum and the vertical limb of the diagonal band. In particular, ChAT-positive fibers coursing throughout the septum and cingulum, which presumably arose from medial septal neurons, were more intensely stained in the T_3-treated males than in the control males, the control females, or the T_3-treated females (Fig. 4). In addition these axons appeared to possess larger and more varicosities than ChAT-immunoreactive fibers in comparable brain regions of other treatment groups (Fig. 4).

Effects of T_3 on NGF-R immunohistochemistry. Like the observed effects of T_3 on ChAT-positive neurons, staining for NGF-R was markedly different in the T_3-treated males compared with all other groups; NGF-R-positive fibers coursing dorsally in the medial septum showed larger and more varicosities (Fig. 5). Hippocampal NGF-R-immunoreactivity was substantially more intense in the T_3-treated males than in the control males (Fig. 5). No differences in the intensity of hippocampal staining were detectable between female control and T_3-treated rats. No distinct variability in NGF-R immunoreactivity was observed among untreated controls or between treated and untreated females.

DISCUSSION

This study has revealed marked sex differences in the qualitative and quantitative effects of neonatal T_3 treatment on the septohippocampal cholinergic system. T_3 treatment of neonatal male rats results in an increase in ChAT activity and muscarinic receptor binding later in life. The treatment also causes an increase in the density of ChAT-immunoreactive cell bodies concom-

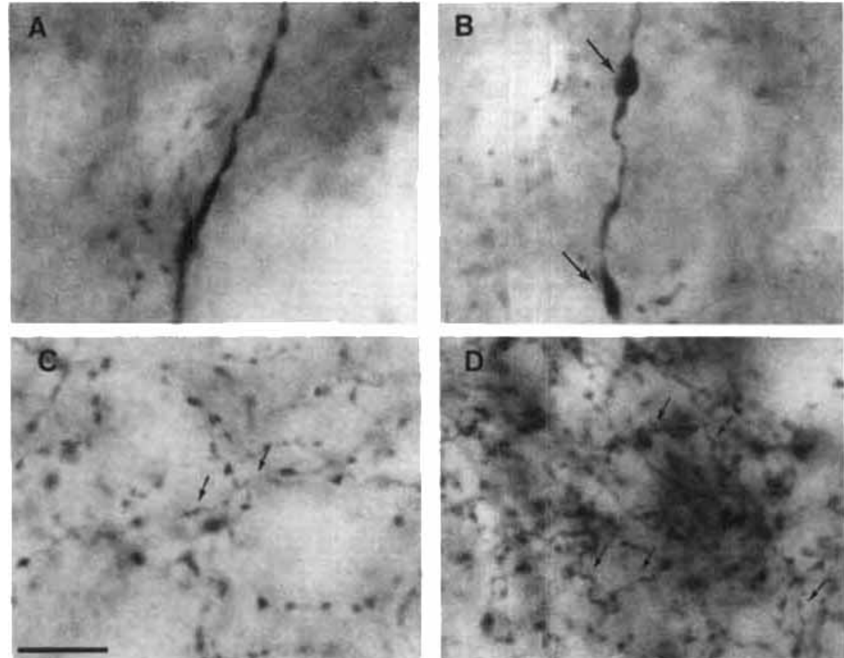

FIG. 5. Representative photomicrographs of NGF-R immunoreactive fibers in the medial septum (**A, B**) and hippocampus (**C, D**) of control (A, C) and T_3-treated (B, D) male rats. Observe the increase in the size of varicosities in B compared with A. Note the increase in immunoreactivity in D compared with C. Arrowheads in B indicate varicosities, those in C and D point to the fibers. Scale bar in C equals 10 μm and applies to all frames.

itant with a decrease in the cross-sectional cell area. In contrast, T_3-treated females showed an increase in the cross-sectional area of ChAT-immunoreactive cell bodies, but none of the other variables changed significantly. Another sex difference, which again emphasizes the paucity of response in females, was the enhanced intensity of ChAT-immunoreactive staining in septal–diagonal band cell bodies and fiber tracts of T_3-treated males. Both size and number of ChAT-immunoreactive varicosities increased as well as the thickness of the fibers themselves. A similar enhancement of NGF-R immunoreactivity by T_3 treatment was seen in males.

The parallel rise in ChAT activity and ChAT-immunoreactive staining seen in the septum–diagonal band of T_3-treated male rats may be explained by the appearance of additional numbers and increased size of ChAT-positive varicosities along with an overall increase in the number of ChAT molecules per neuron. ChAT-positive varicosities are known to form synaptic contacts, and contain synaptic vesicles where ChAT immunoreactivity is concentrated, as evidenced by immunocytochemical studies in the rat cerebral cortex (Houser et al., 1985). Also, in cell fractionation studies, the highest ChAT enzyme activity, by far, is recovered in the synaptosomal fraction (Tuček, 1988).

In contrast, the hippocampus does not exhibit changes in ChAT activity following neonatal T_3 treatment. This is surprising in view of the fact that neurons that give rise to the septal projection are both biochemically and histochemically altered and hippocampal NGF-R immunoreactivity is more intense in T_3-treated males than in matched controls. It is conceivable that the increase in size and number of ChAT-positive varicosities along with the increase in the number of ChAT molecules are counterbalanced by a reduction in the total size of the cholinergic terminal axonal field, giving rise to a normal level of ChAT enzyme activity. Because changes in cell body size can reflect alterations in the axonal fields (O'Kusky, 1985; Sofroniew et al., 1987), the almost 50% decrease in cell body area in the medial septum of the T_3-treated males is consistent with this possibility. Septal cholinergic cells (Swanson and Cowan, 1976; Milner et al., 1983) are formed prenatally, leaving subsequent postnatal synaptogenesis and fiber regression available for thyroid hormone influence. A recent study of the development of the septohippocampal projection in postnatally hypothyroid rats (Rami et al., 1989) showed a transient decrease in acetylcholinesterase (AChE, EC 3.1.1.7) activity and AChE histochemical reaction product in the hippocampal formation. One explanation was that the hippocampus was hyperinnervated by the septal projection after treatment had ceased, virtually restoring the AChE levels to their normal values. As early hypo- and hyperthyroidism frequently produce opposite effects (Patel and Balazs, 1980), this would agree with our suggestion that hypoinnervation of the hippocampus may be the final result of excess T_3 during development.

A number of mechanisms may be involved in the increase in muscarinic receptor numbers in the septal–diagonal band region and in the dorsal hippocampus of T_3-treated males. Thus, the increment in cholinergic varicosity density may well be accompanied by an increased formation of postsynaptic elements accommodating muscarinic receptors. Alternatively, elevated muscarinic receptor numbers may represent supersensitivity in response to the hypothesized septal fiber hypoinnervation of the hippocampus discussed above. The possibility that elevated glial cell numbers may account, at least partially, for increased muscarinic re-

ceptor density cannot be excluded, as muscarinic receptors have been demonstrated in purified cultures of glial cells from chick brain (Repke and Maderspach, 1982). A rise in both ChAT activity and muscarinic receptors has been shown in vitro for reaggregate cell cultures from fetal whole forebrain (Atterwill et al., 1984). Finally, in all our neurochemical studies, the tissue dissection used for the neurochemical studies (see Fig. 1) undoubtedly included bits of caudate-putamen tissue which would have added to total cholinergic activity measured; however, this likely contributed to a systematic error and does not detract from the main findings, given the morphological results, which are anatomically precise (Hoover et al., 1978; Rotter et al., 1979).

An important factor that may be involved in producing the sex differences demonstrated here is timing of cholinergic development. Because the result of early T_3 action seems to be a function of the target neuron's maturational disposition, asynchronous development of the septohippocampal complex across sexes may well lead to sex differences. Therefore, it is interesting that the development of cholinergic indices in the female rat dentate gyrus and septum, as measured by ChAT activity, has been found to precede that in the male (Loy and Sheldon, 1987). Moreover, significant sex differences in thyroid function do exist during postnatal development (Gayo et al., 1986). The time of onset of thyroid function may be critical in defining the temporal characteristics of brain development.

Whether sex steroids are directly involved in influencing the development of the septal–diagonal band cholinergic neurons is not yet known. However, it is notable that testis weight shows a marked decline following neonatal hyperthyroidism (Bakke et al., 1975). This fact introduces the possibility that testosterone levels are lower in T_3-treated rats than in controls. Attenuated testosterone levels could result in a lack of androgenization of the septal–diagonal band neurons. This may be one explanation for the apparent "feminization" of some of the morphological traits of these neurons seen in the T_3-treated male rats.

Both T_3 and nerve growth factor (NGF) have been shown to promote ChAT activity in cell culture, seemingly by different mechanisms, as their synergistic actions result in a more than additive increase in enzyme activity (Hayashi and Patel, 1987; Patel et al., 1988). Several studies support the notion that NGF, released from cholinergic target cells, binds to specific receptors (NGF-Rs) and is retrogradely transported to cholinergic cell bodies where ChAT activity is stimulated (Schwab et al., 1979; Seiler and Schwab, 1984). Early thyroid hormone treatment leads to increased levels of NGF in the neonatal mouse brain (Walker et al., 1981) and, although conclusive evidence is lacking, it has been proposed that T_3 may stimulate the production of NGF-Rs (Bernd and Greene, 1984). Our findings may reflect an elevated overall synthetic activity in the NGF/NGF-R system in the septohippocampal complex which could account for increased cholinergic activity. It remains to be clarified whether the developmental effects of T_3 on the neonatal rat brain are partially mediated by NGF.

A sexually selective action of thyroid hormone on the development of the septohippocampal system is manifested in this study. Because unimpaired septal function is imperative for normal hippocampal performance, the basis for sexual disparities that may become apparent in spatial or stress behavior, cognitive abilities, or even neuropathological processes may, at least in part, lie in the differential susceptibility of septal–diagonal band cells to early influence of thyroid hormones.

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