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Phenylethanolamine *N*-methyltransferase-containing neurons in the limbic system of the young rat

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ABSTRACT **Fifteen years ago epinephrine cells were shown to be present in the medulla oblongata of the rat. These cell groups (C1 and C2) were thought to supply the epinephrine innervation in the rest of the central nervous system. In this study I demonstrate the presence of epinephrine-producing neurons in the forebrain of the young rat. Neurons that are immunopositive for phenylethanolamine *N*-methyltransferase (*S*-adenosyl-L-methionine:phenylethanolamine *N*-methyltransferase, EC 2.1.1.29) are present in the central nucleus of the amygdala as well as in the bed nucleus of the stria terminalis. Neurons in the same location are also immunopositive for tyrosine hydroxylase [tyrosine 3-monooxygenase; L-tyrosine, tetrahydrobiopterine:oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2]. The phenylethanolamine *N*-methyltransferase immunopositivity disappears by day 35, while a small amount of tyrosine hydroxylase-positive cells still can be found in the adult. *In situ* hybridization reveals tyrosine hydroxylase mRNA in the above nuclei in both young and adult animals. The number of the positive cells decreases in adulthood. RNA blot-hybridization analysis showed the presence of phenylethanolamine *N*-methyltransferase mRNA in the amygdala and the bed nucleus of the stria terminalis in the young and in the adult rat brain. Neurons that are immunopositive for phenylethanolamine *N*-methyltransferase are also present in the human amygdala.**

Dahlström and Fuxe, using the formaldehyde-induced fluorescence method, first described the distribution of catecholamines in the rat central nervous system (CNS) in 1965 (1). Subsequently, others have used antibodies against enzymes involved in catecholamine biosynthesis to map cells that produce dopamine, norepinephrine and epinephrine (see ref. 2). Some years after Axelrod discovered phenylethanolamine-*N*-methyltransferase (PNMTase; *S*-adenosyl-L-methionine:phenylethanolamine *N*-methyltransferase, EC 2.1.1.29), the enzyme that converts norepinephrine to epinephrine (3), Hökfelt *et al.* demonstrated its presence in the brain immunocytochemically (4, 5).

Epinephrine is widely distributed in the CNS, and two groups of cells were thought to supply it to the rest of the brain and spinal cord: C1 and C2 cell groups in the medulla oblongata. Recently, however, a group of PNMTase-positive cells has been found in the posterior hypothalamus (6), that lack the other catecholamine-synthesizing enzymes. Clearly these neurons are different from the C1 and C2 cells. If they make epinephrine, they must do so by taking up norepinephrine first.

Here I report the presence of PNMTase-immunopositive neurons in the central nucleus of the amygdala and in the bed nucleus of the stria terminalis in young rats (from birth to 35 days). I also show the presence of tyrosine hydroxylase in the above areas by means of immunocytochemistry and the presence of tyrosine hydroxylase [tyrosine 3-monooxygenase; L-tyrosine, tetrahydrobiopterine:oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2] mRNA in the amygdala in the young and in the adult rats by *in situ* hybridization histochemistry. RNA blot-hybridization (Northern) analysis of brain samples confirms the finding of PNMTase mRNA in the amygdala and the bed nucleus of the stria terminalis.

PNMTase and tyrosine hydroxylase-immunopositive neurons are found in the human as well as in the rat amygdala.

The above findings suggest that in addition to the C1 and C2 epinephrine-containing cells, there are at least two more adrenergic populations. Because of technical difficulties (the need to use free-floating sections), I was unable to demonstrate the colocalization of tyrosine hydroxylase and PNMTase in the very same cells in adjacent sections. However, since tyrosine hydroxylase is also present in the amygdala cells in the same distribution with PNMTase, it is likely that the same cells contain all of the enzymes involved in the synthesis of catecholamines, unlike the posterior hypothalamic cells (6) that contain PNMTase exclusively.

MATERIALS AND METHODS

Immunocytochemistry. Male Sprague–Dawley rats were killed (a total of 60; 2 or 3 rats at a timepoint) every other day from day 1 to day 35. Rats were anesthetized with ether and perfused through the heart with 4% paraformaldehyde/picric acid in 0.127 M sodium phosphate buffer (pH 7.4). The brains were removed and soaked in fixative overnight. They were mounted on specimen blocks and embedded in low-melting agar (1.5%) to facilitate sectioning. Sections (50–80 μm thick) were cut on a Vibratome. The sections were washed in phosphate-buffered saline (pH 7.4), endogenous peroxidase activity was blocked with 3% H_2O_2 for 30 min, the sections were washed again, and incubation with the primary antibodies for 48 hr at 4°C using the free-floating technique was carried out. The sections were then washed and transferred into a solution of biotinylated anti-rabbit IgG (1:400 dilution) for 1 hr at room temperature. After this wash, the sections were incubated in horseradish peroxidase-conjugated avidin–biotin complex (1:200) (Vector Laboratories) for 1 hr at room temperature and were developed by using 3,3′-diaminobenzidine tetrahydrochloride as a substrate. The sections were gently shaken during all of the above steps. Then they were transferred onto gelatin-coated slides, dehydrated, and mounted in plastic mounting medium. The stained sections were studied with a Leitz Dialux microscope with a blue gelatin filter to increase contrast.

Human brains were put into the above fixative solution 6–10 hr postmortem. The amygdala was dissected the following day and left in fixative for an additional 2–3 days. At that point the tissue was mounted, cut on a Vibratome, and treated as described earlier.

Three rabbit anti-PNMTase polyclonal antisera were used from two sources: (*i*) Eugentec lots 3011 (1984) and 3012

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Abbreviations: CNS, central nervous system; PNMTase, phenylethanolamine *N*-methyltransferase.

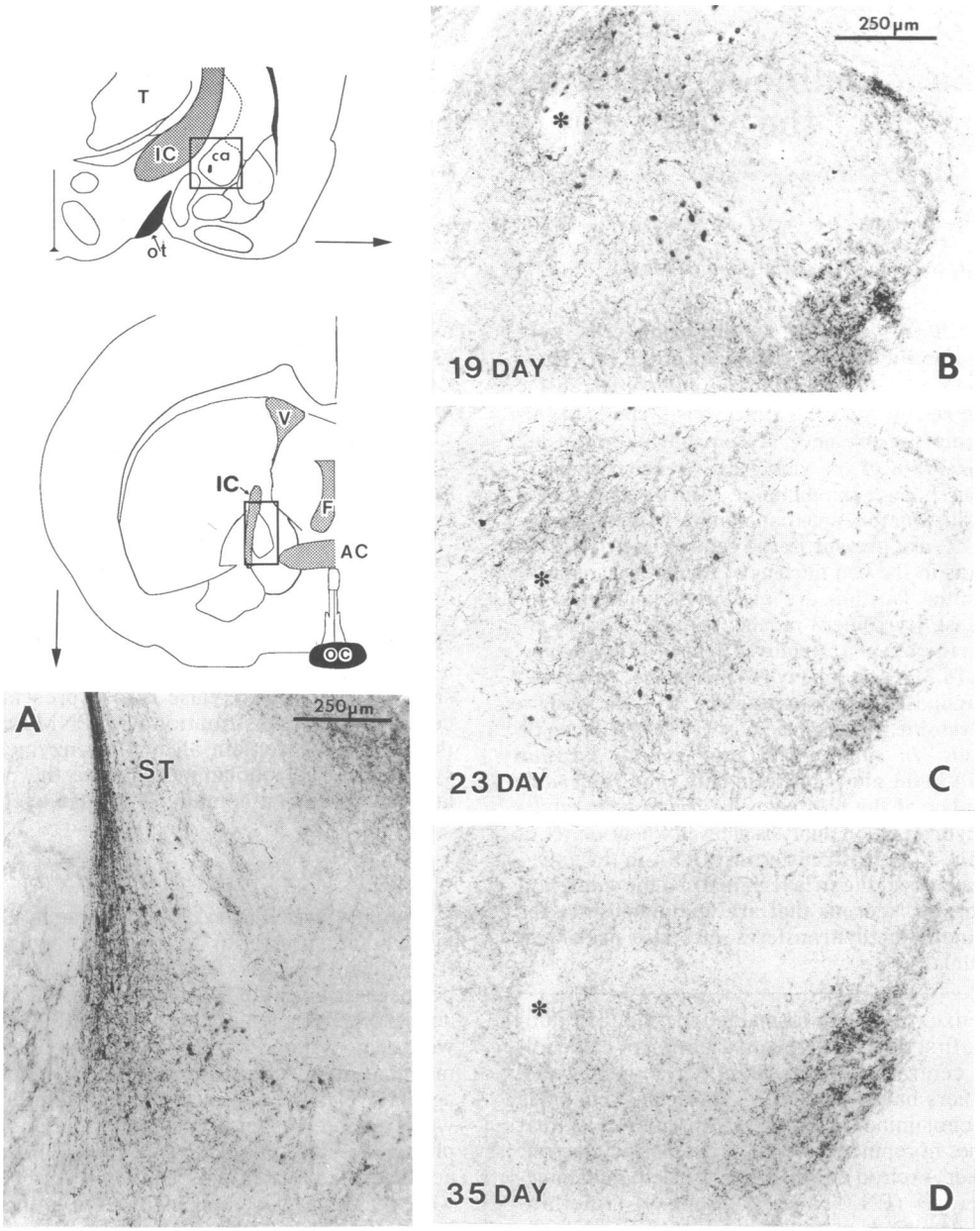

FIG. 1. PNMTase immunostaining in the bed nucleus of the stria terminalis (*A*) and in the central nucleus of the amygdala (*B–D*) of the rat. Immunopositive cells are shown in *A* (19-day-old rat) and in *B* and *C* (19- and 23-day-old rats). Note the disappearance of the cell bodies at 35 day (*D*). The boxes in the schematic drawings indicate the areas shown in the figures. The asterisks in *B*, *C*, and *D* indicate the stria terminalis. The intensely innervated area in the lower right portion is the basal amygdaloid nucleus that was reported to contain the highest amounts of epinephrine in the amygdala (15). AC, anterior commisure; ca, central amygdaloid nucleus; F, fornix; IC, internal capsule; oc, optic chiasm; OT, optic tract; ST, stria terminalis; T, thalamus; V, lateral ventricle.

(1986) and (*ii*) a third serum from M. Goldstein (New York University Medical Center) (see refs. 4 and 5). All three antibodies were used in a 1:1000 dilution. Antibodies directed against tyrosine hydroxylase were purchased from Eugentec (lots 1011 and 1012), and one was a gift of T. Joh (Cornell University Medical College) (7). These antibodies were used in a 1:1000 dilution except for the last one, which was used at 1:2500. All dilutions were made in phosphate-buffered saline containing 0.1% bovine serum albumin (Miles Pentex V.) and 0.6% Triton X-100. All of the figures show sections that were stained with Eugentec PNMTase and Joh's tyrosine hydroxylase antibodies.

As an absorption control, the PNMTase antibody at a 1:1000 dilution was incubated with 30 μg of purified PNMTase (Sigma) per ml overnight at 4°C and then was used to stain sections.

***In Situ* Hybridization Histochemistry.** Rats were decapitated, and their brains were removed and frozen on dry ice. Sections (12 μm thick) were cut in a cryostat and mounted on gelatin-coated slides. The tyrosine hydroxylase probe was directed towards bases 1441–1488 of the tyrosine hydroxylase mRNA (8) and was radioactively labeled (^{35}S) as described (9). The sections then were dipped in Kodak NTB3 emulsion, developed after 3 weeks, and examined under dark-field illumination.

Northern Blot Analysis. Adult (200 g) and 14-day-old rats (30 rats each group) were decapitated, and brain regions were dissected immediately from unfrozen tissue slices by the punch method (10). The samples were frozen on dry ice, and tissues collected from 30 rats were pooled. The pooled samples were homogenized in a glass homogenizer in 2 ml of 5.5 M guanidine thiocyanate (Eastman Kodak)/25 mM so-

dium citrate, pH 7.0/0.5% *N*-laurylsarcosine/0.2 M 2-mercaptoethanol (final concentration; prepared just before use). The RNA was recovered by a single-step extraction procedure (11). Poly(A)$^+$ RNA was isolated with oligo(dT)-cellulose (12). The poly(A)$^+$ RNA was then reconstituted in denaturing buffer, and the samples were run in a denaturing 1% agarose gel. The RNA was then stained with ethidium bromide and destained overnight, and pictures of the gels were taken. The RNA was then transferred to nitrocellulose (GeneScreen*Plus*) via electrophoresis for 4 hr. The blot was baked for 2 hr at 80°C to fix the RNA. Next the blot was incubated in hybridization solution at 42°C for 5 hr, then radiolabeled cDNA probes were added to the solution, and the blots were hybridized overnight (for more details, see ref. 13). After hybridization, the blots were washed and placed against Kodak XAR3 film with intensifying screens. Rat adrenal medullary RNA was run next to the samples as a control.

The probes were prepared by labeling a large fragment of the DNA complementary to PNMTase mRNA that had been cloned from an adrenal medullary cDNA library (a generous gift of Hiroto Okayama, National Institute of Mental Health, Bethesda, MD) by using the bovine PNMTase sequence (14) for screening (unpublished data). The cDNA was labeled by random primer labeling and applied at 10^6 cpm/ml in hybridization solution.

RESULTS

Immunocytochemistry in the rat brain revealed PNMTase and tyrosine hydroxylase-immunopositive neurons in the central nucleus of the amygdala and in the lateral and dorsal portions of the bed nucleus of the stria terminalis (Figs. 1 and 2). The number of these catecholamine-positive neurons seemed to increase until day 14, and then there was no significant change in their number up to about day 24. Subsequently, there was a gradual decline in their number. The rate of decline increased between days 30 and 35, and by day 35 there was practically no detectable PNMTase-immunoreactive neuron left in the amygdala or the bed nucleus of the stria terminalis. A few tyrosine hydroxylase-positive neurons occasionally were seen in the adult rat brain amygdala (Fig. 3). In the stria terminalis of the adult rat brain, there were both PNMTase- and tyrosine hydroxylase-immunopositive fibers. Preabsorbing the diluted PNMTase antibody with purified PNMTase abolished the immunostaining.

In the human amygdala, both PNMTase- and tyrosine hydroxylase-immunopositive neurons were present (Fig. 4). Cell bodies were located in the basal and central nuclei, and the former also had a dense network of PNMTase-positive fibers.

In situ hybridization revealed the presence of tyrosine hydroxylase mRNA in the cells of the central nucleus of the amygdala. In the young rat there were more neurons (four or five in one 12-μm thick section) than in adult cells, which had zero to two per section (Figs. 3 and 5). Occasionally I also could detect individual cells in the bed nucleus of the stria terminalis that were positive when hybridized with the

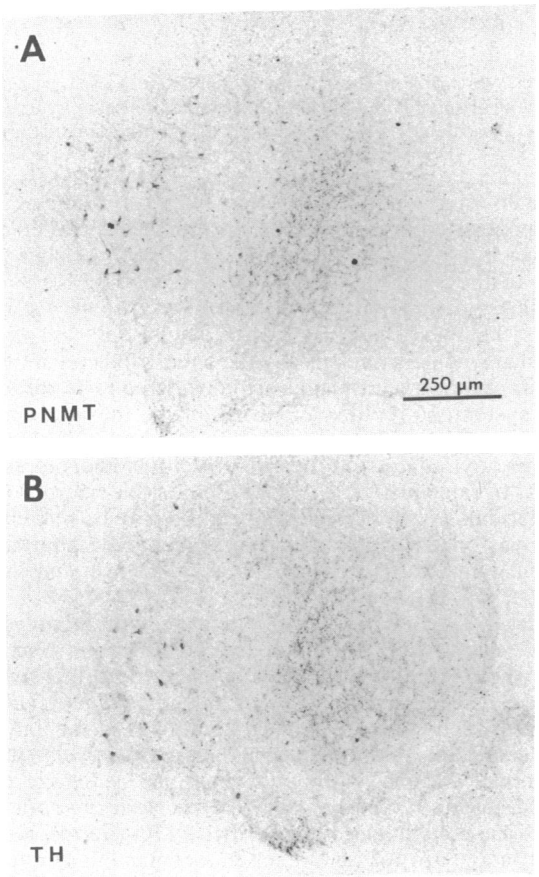

FIG. 2. PNMTase (PNMT) and tyrosine hydroxylase (TH) immunostaining in 7-day-old rat amygdala in 50-μm-thick Vibratome sections. The distribution of the two antigens in cell bodies and fibers is similar.

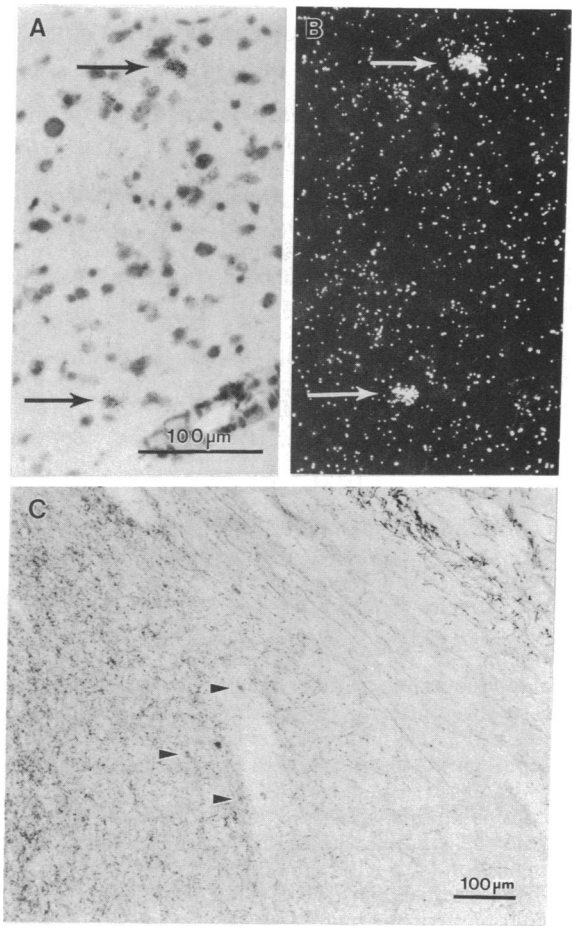

FIG. 3. *In situ* hybridization cytochemistry shows the presence of tyrosine hydroxylase mRNA in cells of the adult rat central amygdala in bright-field illumination (*A*) and the same area with dark-field illumination (*B*). A few faint tyrosine hydroxylase-immunopositive cells can still be seen in the central nucleus of the amygdala (arrowheads in *C*).

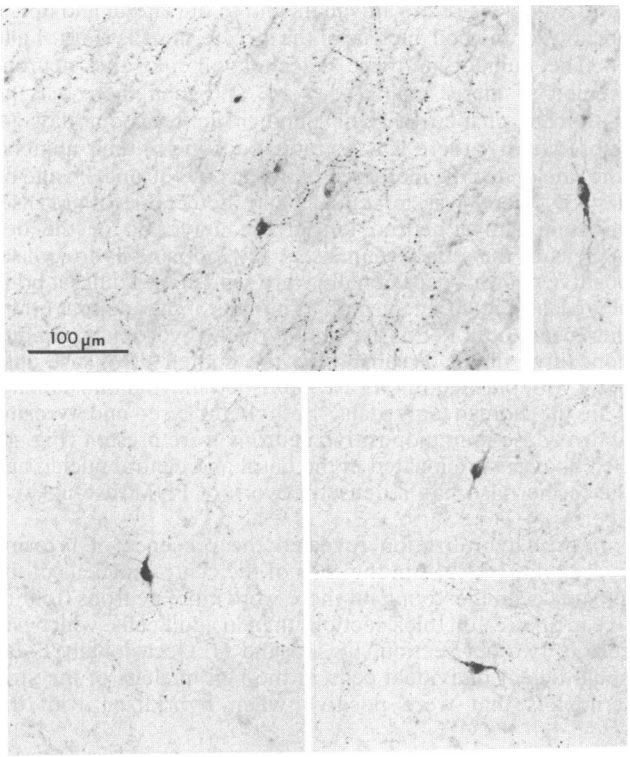

FIG. 4. PNMTase-immunopositive neurons in the human amygdala.

tyrosine hydroxylase probe. Our method did not seem to be sensitive enough to show PNMTase mRNA in these cells.

Northern blot analysis showed the presence of a mRNA in the amygdala of 14-day-old rats that hybridized to the PNMTase cDNA probe. The size of this message was around 1.4 kilobases (kb) and corresponded to the positive band found in the rat adrenal medulla. In the adult rat brain, the band was still visible, but the signal was much fainter than in young animals. In the young rats, there seemed to be an additional band that was around 2 kb that hybridized to the PNMTase cDNA used. This band was not detected in adult brain extracts under our conditions.

DISCUSSION

Epinephrine was first detected in the mammalian CNS by bioassay in 1954 (16); this finding was later supported by several groups using biochemical techniques (see ref. 17). Because of its low concentration in the CNS brain, epinephrine was ignored while other catecholamines (dopamine and norepinephrine) were widely studied. In addition, since formaldehyde-induced fluorescence did not permit investigators to differentiate between norepinephrine and epinephrine, we had a very limited knowledge of the localization of CNS epinephrine neurons. Until immunohistochemistry enabled workers to visualize PNMTase-containing neurons, the connections and possible functional role of adrenergic cells could not be studied. Since the first immunocytochemical demonstration (4, 5), the connection and functions of these cells have been exhaustively studied (see refs. 2 and 17). Epinephrine neurons have been suggested to play a role in regulating blood pressure (17, 18), respiratory control (17) and neuroendocrine regulation (17, 19–22) including possible growth hormone regulation (23). Finally, several studies demonstrated behavioral effects of PNMTase inhibitors (17).

The epinephrine-containing neurons in the central amygdaloid nucleus and in the dorsolateral bed nucleus of the stria terminalis comprise a new catecholamine-producing neuronal population in the CNS. The presence of PNMTase and tyrosine hydroxylase in the same population of cells (very likely in the same cells) and the presence of the mRNA encoding tyrosine hydroxylase and PNMTase very strongly suggest that these cells in fact are producing epinephrine. The central amygdaloid nucleus and the lateral division of the bed nucleus of the stria terminalis are considered to be part of the same system. In reptiles and amphibians, they are actually part of the same nuclear mass (24). In mammals this is not that obvious anymore, since the two structures become discontinous. In rats clusters of PNMTase-positive neurons in the stria terminalis can be observed. These may be remnants of what was once a single neuronal system. The afferent and efferent connections of the two areas are also very much alike (25).

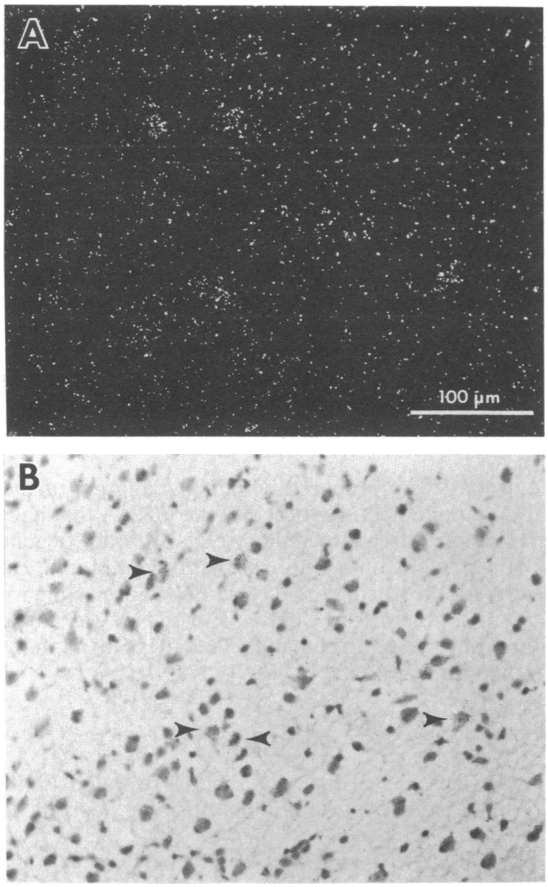

FIG. 5. *In situ* hybridization histochemistry shows several cells in the central amygdaloid nucleus that contain tyrosine hydroxylase mRNA. Dark-field (*A*) and bright-field (*B*) illumination of the same area.

Tract tracing studies showed that the central amygdaloid complex has reciprocal connections with brain stem areas (26), where the already known epinephrine cell groups are located. It seems likely that these amygdaloid PNMTase cell groups, which could be called C4 and C5* in the amygdala and bed nucleus of the stria terminalis, respectively, are part of the same PNMTase-producing neuronal systems as the C1 and C2 groups in the brainstem. The posterior hypothalamic PNMTase-containing cells described by Ruggiero *et al.* (6) do not contain any of the other catecholamine-synthesizing enzymes and probably should not be designated a "C" group. The role of the adrenergic cells designated C4 and C5

*A few epinephrine-positive neurons that are the most medial cells of the C2 cell group were designated C3 (27).

is unknown. Since they significantly reduce their production of catecholamines at the time of sexual maturity, they may be involved in puberty. They may also function in growth or development of the CNS. The factors that control the level of catecholamine production in these cells are not known and should be investigated. Whether these cells' loss of PNMTase is permanent or can be reversed in adult animals is also unknown.

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1. Dahlström, A. & Fuxe, K. (1964) *Acta Physiol. Scand. Suppl.* **232,** 1–55.
2. Hökfelt, T., Johansson, O. & Goldstein, M. (1984) in *Handbook of Chemical Neuroanatomy*, eds. Björklund, A. & Hökfelt, T. (Elsevier, Amsterdam), Vol. 2, pp. 157–276.
3. Axelrod, J. (1962) *J. Biol. Chem.* **237,** 1657–1660.
4. Hökfelt, T., Fuxe, K., Goldstein, M. & Johansson, O. (1973) *Acta Physiol. Scand.* **89,** 286–288.
5. Hökfelt, T., Fuxe, K., Goldstein, M. & Johansson, O. (1974) *Brain Res.* **66,** 235–251.
6. Ruggiero, D. A., Ross, C. A., Anwar, M., Park, D. H., Joh, T. H. & Reis, D. J. (1983) *J. Comp. Neurol.* **239,** 127–154.
7. Armstrong, D. M., Ross, C. A., Pickel, V. M., Joh, T. & Reis, D. J. (1982) *J. Comp. Neurol.* **212,** 173–187.
8. Grima, B., Lamouroux, A., Blanot, F., Biguet, N. F. & Mallet, J. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 617–621.
9. Young, W. S., Warden, M. & Mezey, E. (1987) *Neuroendocrinology* **46,** 439–444.
10. Palkovits, M. (1973) *Brain Res.* **59,** 449–450.
11. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
12. Jacobson, A. (1987) *Methods Enzymol.* **152,** 254–261.
13. Fischer-Colbrie, R., Iacangelo, A. & Eiden, L. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 3240–3244.
14. Baetge, E. E., Suh, Y. H. & Joh, T. H. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 5454–5458.
15. Van Der Gugten, J., Palkovits, M., Wijnen, H. L. S. M. & Versteeg, D. H. G. (1976) *Brain Res.* **107,** 171–175.
16. Vogt, M. (1954) *J. Physiol. (London)* **123,** 451–481.
17. Fuller, R. W. (1982) *Annu. Rev. Pharmacol. Toxicol.* **22,** 31–55.
18. Petty, M. A. & Reid, J. L. (1979) *Brain Res.* **163,** 277–288.
19. Fuxe, K., Andersson, K., Agnati, L. F., Ferland, L., Hökfelt, T., Eneroth, P., Gustafsson, J. A. & Skett, P. (1979) in *Catecholamines: Basic and Clinical Frontiers*, eds. Usdin, E., Kopin, J. & Barchas, J. (Pergamon, New York), pp. 186–188.
20. Crowley, W. R. & Terry, L. C. (1981) *Brain Res.* **204,** 231–235.
21. Roth, K. A., Katz, R. J., Sibel, M., Mefford, I. N., Barchas, J. D. & Carrol, B. J. (1981) *Life Sci.* **28,** 2389–2394.
22. Mezey, E., Kiss, J. Z., Skirboll, R. L., Goldstein, M. & Axelrod, J. (1984) *Nature (London)* **310,** 140–141.
23. Gold, M. S., Donabedian, R. K. & Redmond, D. E., Jr. (1978) *Psychoneuroendocrinology* **3,** 187–194.
24. Johnston, J. B. (1923) *J. Comp. Neurol.* **35,** 337–481.
25. DeOlmos, J., Alheid, G. F. & Beltramino, C. A. (1985) in *The Rat Nervous System*, ed. Paxinos, G. (Academic, New York), Vol. 1, pp. 223–334.
26. Schwaber, J. S., Kapp, B. S., Higgins, G. A. & Rapp, P. R. (1982) *J. Neurosci.* **2,** 1424–1438.
27. Howe, P. R. C., Costa, M., Furness, J. B. & Chalmers, J. P. (1980) *Neuroscience* **5,** 2229–2238.