NEUROGENESIS OF CATECHOLAMINE NEURONS IN THE MOUSE BRAIN STEM

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ABSTRACT

The time course of production of catecholamine neurons in the mouse brain stem was examined by [3 H]thymidine autoradiography and peroxidase-antiperoxidase (PAP)-immunocytochemistry applied simultaneously to the same tissue section. To identify catecholamine neurons, an antiserum for tyrosine hydroxylase (TH) was used. The period of neurogenesis of TH-immunoreactive neurons was embryonic day 9-11 (E9-E11) in the lateroventral reticular formation of the medulla oblongata (A1), E9-E12 in the nucleus tractus solitarii complex (A2), E9-E11 in the locus coeruleus (A6), E10-E13 in the substantia nigra (A9) and the ventral tegmental area (A10), and E11-E15 in the hypothalamic arcuate nucleus (A12). The peak of neurogenesis was seen in E10 for A1 and A6, in E11 for A2, A9 and A10, and in E13-E14 for A12. The results indicate that noradrenergic neurons (A1, A2, A6) begin to be produced earlier than dopaminergic neurons (A9, A10, A12). Among the noradrenergic neurons, A2 cells differentiated later and took a longer period than A1 or A6 cells. Most of the hypothalamic A12 cells were produced after the midbrain dopaminergic cells (A9, A10) had differentiated. The data indicate that the neurogenesis of catecholamine neurons occurs in varying time sequence according to their location in brain and begins several days earlier than the phenotypic expression of catecholamines.

For a better understanding of development of the catecholamine neuron system in the brain, four basic problems must be elucidated; 1) the sequence of neurogenesis of catecholamine neurons from their matrix cells, 2) the time course of production of neurons that synthesize transmitter catecholamines or of catecholamine-synthesizing enzymes (*i.e.* tyrosine hydroxylase or dopamine- β -hydroxylase), 3) the guidance of catecholamine neurons to their target organs (*e.g.* formation of nigro-striatal pathways), and 4) the formation of morphological and functional synapses on target neurons.

Although the guidance sequence of catecholamine neurons to their target organs has not yet been elucidated, the other problems have been studied by various methods. Many

findings have been obtained on the appearance and development of catecholamines and their synthesizing enzymes in the brain by biochemical (1, 10-12) and morphological (9, 16, 17, 24, 30, 35, 40, 45, 46) techniques. The developmental sequence of synaptic formation in target organs has been studied in nigro-striatal system (48) and the cerebral cortex (13).

In 1958, Hughes *et al.* (23) synthesized tritium-labeled thymidine of high specific activity and demonstrated that the thymidine incorporated into the synthetic phase of DNA remained there as a fixed component during the life span of the cell. Sauer and Walker (39) and Sidman *et al.* (44) were the first to apply this method for analyzing the time course of neurogenesis. By using this technique, matrix

cells have been proven to synthesize DNA and go through mitosis and also become neuroblasts (18, 19). The administration of [^3H]thymidine on an indicated day of embryonic life enables us to determine the time of final mitotic division of matrix cells. Several autoradiographic studies have been made on the developing nervous systems and the time of neuron production in various brain regions has been determined almost completely in the mouse and the rat (2-7, 25, 41, 47).

However, ordinary [^3H]thymidine autoradiography can not demonstrate the time of production of neurons with specific phenotypes. Thus, only limited information is available on the sequence of genesis of catecholamine neurons. Lauder and Bloom (28) demonstrated monoamine neuron production in serial sections by the alternate application of autoradiography and the Falck-Hillarp histofluorescence technique. Rothman *et al.* (38) applied [^3H]thymidine autoradiography and tyrosine hydroxylase immunohistochemistry in the same tissue section of embryonic animals and found that tyrosine hydroxylase was expressed after the neurons were born from matrix cells. However, they did not analyze the time course of catecholamine neuron production in various brain nuclei. A complete study of the development of various catecholamine neuron systems in a mammal has not yet been done. We studied the patterns of neurogenesis of major catecholamine neuron systems in the mouse brain by [^3H]thymidine autoradiography and PAP immunocytochemistry applied to the same tissue section. This method has been valuable for analyzing the time course of production of neurons containing other transmitters, including somatostatin (21), vasopressin (32), oxytocin (32), endogenous opiates (27), GABA (15) and vasoactive intestinal peptide (VIP) (34). The mouse was chosen as the experimental animal for several reasons. The mouse is one of the most commonly studied animals in mammalian embryology. Besides, there are many strains of mutant mice with abnormalities of the central nervous system (37, 43), and they can be studied and compared with the normal data provided by this study.

MATERIALS AND METHODS

The animals used in this experiment were pregnant ICR mice. The methods used here were as previously described (33).

The day when a vaginal plug was observed was defined as embryonic day 1 (E1). The pregnant dams were subcutaneously injected with [^3H]thymidine (specific activity, $>15\text{ Ci/mmole}$, New England Nuclear) twice on the same day in a total dose of $7.5\text{ }\mu\text{Ci/g}$ body weight (first $5\text{ }\mu\text{Ci/g}$ body weight, then $2.5\text{ }\mu\text{Ci/g}$ body weight). The time interval between the first and the second injection of the radio isotope was decided based on matrix cell kinetics (26); *i.e.*, 4 h on day E8 and E9, 4.5 h on E10, 5 h on E11 and E12, 7 h on E13, E14, E15 and E16, and 9 h on E17. Injected dams continued their pregnancies to delivery on E20. The progeny of injected dams were sacrificed at 8-10 weeks postnatally by cardiac perfusion with a fixative containing 4% paraformaldehyde, 0.2% picric acid and 0.35% glutaraldehyde in phosphate buffer. After postfixed with the fixative without glutaraldehyde, brains were cut into $6\text{ }\mu\text{m}$ sections using a cryostat. Then the sections were processed for immunocytochemistry by a modified double bridge PAP method (33). Sections were incubated in diluted anti-tyrosine hydroxylase (TH) serum (1:5,000) for 12 h at room temperature. The specificity and characteristics of the TH antiserum have been described elsewhere (31). Sections were then soaked in a solution of goat anti-rabbit IgG (Miles) and then peroxidase antiperoxidase (PAP) complex (Dako). After each of these steps, sections were rinsed with 0.1 M phosphate-buffered saline. Then the processes for anti-rabbit IgG and PAP were repeated. Finally, the sections were exposed to 0.02% 3,3'-diaminobenzidine-4HCl (DAB) (Wako) solution, containing 0.03% H_2O_2 . Then the sections were coated with Ilford L4 emulsion by the dipping method, and exposed for 4 weeks in a black box. The exposed film was developed with Fuji FD 111.

Combination of immunocytochemistry and [^3H]thymidine autoradiography enables us to detect TH-immunoreactive neurons with brown-colored immunoreactivity in their cytoplasm and silver grains on each nucleus. TH-Immunoreactive and non-immunoreactive neurons were counted: neurons with more than half of the maximum number of silver grains were regarded as heavily labeled neurons. Approximately 200-1,000 TH-immunoreactive neurons for each catecholamine cell group were counted for statistical analyses. Only neurons which showed an immunoreactive perikaryon with a well-

defined nucleus were counted. The ratio of heavily labeled TH-immunoreactive neurons in each catecholamine cell group was also calculated (Figs. 5 and 6). The nomenclature in this report was according to Sidman *et al.* (42), Paxinos and Watson (36) and Hökfelt *et al.* (22).

RESULTS

As the precise distribution of catecholamine neurons of mouse brain has not been reported before, the terms A1–A12 on the catecholamine neurons in the rat brain named by Dahlström and Fuxe (14) were applied to mouse brain. The anatomical localization of the catecholamine cell groups was quite similar in both species. We describe the time course of production of catecholamine neurons in the lateroventral reticular formation of the medulla oblongata (A1), the locus coeruleus (A6), the substantia nigra (A9), the ventral tegmental area of the midbrain (A10) and hypothalamic arcuate nucleus (A12).

[³H]Thymidine autoradiographic studies combined with tyrosine hydroxylase (TH) immunocytochemistry have revealed the neurogenesis of very small number of catecholamine neurons on E9. No TH-immunoreactive neurons were heavily labeled on E8,

indicating that, the matrix cells undergoing DNA synthesis at this gestational age were labeled, but the labeling was diluted in subsequent divisions before birth. The labeling pattern for each day of injection was described according to the catecholamine cell groups as follows (Figs. 5 and 6).

A1 Cells

At the caudal level of the medulla oblongata, clusters of TH-immunoreactive neurons were present in the ventral and lateral reticular formation (A1). The neurons in this location were distributed largely ventral and lateral to the lateral reticular nucleus. They extended from the caudal border of the medulla oblongata to the caudal portion of the facial nucleus. These neurons were medium to large (long diameter, 13–23 μ m) and multipolar with only a few long processes. Neurons in the A1 cell group were far fewer than those in the locus coeruleus (A6) or in the substantia nigra (A9).

In the animals injected on E9, 3% of the TH-immunoreactive neurons of A1 were labeled. In animals injected on E10, 38% of the TH-immunoreactive neurons were labeled (Fig. 1a). The labeling index of TH-immunoreactive neurons on E11 was 9% (Fig. 5a).

Fig. 1 Coronal sections of animals injected with [³H]thymidine on E10. a: A1 cells in the lateral reticular nucleus of the medulla oblongata. b: A6 cells in the locus coeruleus. c: A9 cells in the pars compacta of the substantia nigra. Note isotope-labeled neurons were detected in A1 and A6 cell groups. In the substantia nigra, however, no labeled immunoreactive neurons were found. Arrows show heavily isotope-labeled immunoreactive neurons. $\times 780$

A2 Cells

TH-Immunoreactive neurons were distributed in the nucleus tractus solitarii complex (A2). The majority of the neurons were located in the dorsal portion of the dorsal vagal motor nucleus and the medial portion of the nucleus of the solitary tract. The neurons were typically smaller (long diameter, 12–19 μm) with less extensive processes. TH-Immunoreactive neurons in the area postrema were not included in the A2 cell group in this report.

In the animals injected on E10, 10% of the TH-immunoreactive neurons of A2 were labeled. The highest labeling index was obtained in animals injected on E11 (26%) (Figs. 2a and 5b).

A6 Cells

TH-Immunoreactive neurons were densely packed in the locus coeruleus (A6), which is the largest noradrenergic neuronal mass in the mouse brain like in the rat brain. In this A6 cell group, the ventral extension of the locus coeruleus, designated 'nucleus subcoeruleus', was included. These TH-immunoreactive neurons were large to medium in size (long diameter, 16–23 μm), and spherical to angular in appearance.

In the animals injected on E9, 3% of the TH-immunoreactive neurons were labeled. These labeled neurons were distributed in the lateroventral border of the nucleus. In the animals injected on E10, 17% of the TH-immunoreactive neurons were labeled and the labeled neurons were distributed in the whole region of the locus coeruleus (Fig. 1b). In the E11-injected group, 7% of the TH-immuno-

reactive neurons were labeled. No labeled TH-immunoreactive neurons were detected in later days (Figs. 3a and 5c).

A9 Cells

In the mesencephalic tegmentum, many TH-immunoreactive neurons in the substantia nigra were defined as A9 cells (14). In the nigra, TH-immunoreactive neurons were mainly confined to the pars compacta, although scattered neurons were distributed in the pars lateralis and pars reticulata. The majority of neurons were medium-sized (long diameter, 14–21 μm).

Only a few TH-immunoreactive neurons were labeled in the animals injected on E10 (Fig. 1c). Labeled TH-immunoreactive neurons were most numerous (23%) in the animals injected on E11 (Fig. 2b). A moderate number of TH-immunoreactive neurons were produced thereafter (E12, 8%; E13, 4%) (Figs. 3b and 6a). The labeling index in the pars compacta was not very different from that in the pars reticulata.

A10 Cells

Between the both sides of the substantia nigra, there was a ventral tegmental area. Many of the TH-immunoreactive neurons distributed in the region were continuous with those distributed in the substantia nigra. The cells were medium-sized (long diameter, 14–20 μm).

The sequence of production of TH-immunoreactive neurons in A10 was similar to that of A9 (Fig. 6b). In animals injected on E11, 11% of the TH-immunoreactive neurons were labeled. Many neurons were labeled in the animals injected on E12 (Fig. 3c) and E13,

Fig. 2 Sections of animals injected with the isotope on E11. a: A2 cells in the nucleus of the solitary tract. b: A9 cells in the pars compacta of the substantia nigra. Arrows show heavily isotope-labeled immunoreactive neurons. Arrowheads indicate isotope-labeled and non-immunoreactive neurons. $\times 500$

Fig. 3 Coronal sections of animals that received the isotope on E12. a: A6 cells in the locus coeruleus. Note no isotope-labeled immunoreactive neurons were detected at this stage. b: A9 cells in the pars reticulata of the substantia nigra. c: A10 cells in the ventral tegmental area of the midbrain. d: A12 cells in the hypothalamic arcuate nucleus. Ependymal cells (double arrows) were labeled at this stage. Arrows indicate heavily isotope-labeled immunoreactive neurons. III, third ventricle. $\times 590$

Fig. 4 Sections of animals injected with the isotope on E14. a: A10 cells in the ventral tegmental area. b: A12 cells in the ventrolateral part of the arcuate nucleus. Note heavily isotope-labeled immunoreactive neurons were still detected in the arcuate nucleus even at this stage. $\times 590$

Fig. 5 Percentage of heavily isotope-labeled TH-immunoreactive neurons in various noradrenergic cell groups in the mouse brain stem. a: A1 cells. b: A2 cells. c: A6 cells

with a labeling index of 9% for both days. Labeled immunoreactive neurons were not detected in the later days (Fig. 4a).

A12 Cells

Among the catecholamine cells in the diencephalon, the hypothalamic arcuate nucleus has the largest number of dopamine cells (A12). These cells were distributed throughout the arcuate nucleus. They were medium-sized (long diameter, 12–15 μm) and had less extensive processes.

The production sequence of the TH-immunoreactive neurons in this nucleus was very different from that of other TH-immunoreactive neurons in the above-mentioned areas (Fig. 6c). Animals injected on E12, E13 and E14 showed 17%, 29% and 28% labeling, respectively (Figs. 3d and 4b). Even in animals injected on E15, 3% of the TH-immunoreac-

Fig. 6 Percentage of heavily isotope-labeled TH-immunoreactive neurons in various dopaminergic cell groups in the mouse brain stem. a: A9 cells. b: A10 cells. c: A12 cells

tive neurons were labeled.

DISCUSSION

The time course of catecholamine neuron production in each cell group (A1, A2, A6, A9, A10 and A12) in mouse brain was examined by application of the combined technique of [^3H]thymidine autoradiography and TH-immunocytochemistry in the same tissue section. By adopting this combination technique, comparison of development of catecholamine neurons in different brain regions is firstly discussed from the aspect of neurogenesis.

We found that the production of noradrenergic neurons (A1, A2 and A6) from the matrix cells begins earlier than dopaminergic neurons (A9, A10 and A12). The maximal production was seen in E10 for A1 and A6, in E11 for A2, A9 and A10, and in E13 for A12. In the noradrenergic cell group, A2 neurons in

the dorsomedial medulla oblongata were produced later and over a longer period. Dopaminergic neurons in the midbrain tegmentum first appeared one day later than the noradrenergic neurons. Although a significant proportion of A10 cells in the ventral tegmental area was produced on E13, there was only a moderate amount of production of nigral A9 cells at this stage. The production of the hypothalamic A12 dopamine cells began on E11, which was one day later than the onset of the production of midbrain dopamine neurons. Production sequence of A12 cells was longer than that of the other catecholamine neurons (A1, A2, A6, A9 and A10). Most of them were produced on E12, E13 and E14. Even on E15, some A12 cells were still produced.

The findings in the present autoradiographic study are roughly in agreement with those in ordinary [^3H]thymidine autoradiographic studies of the brain stem of the mouse (41, 47), although not with those of the lateroventral reticular formation of the medulla oblongata. According to the Taber Pierce's ordinary autoradiographic study (47), neurons in the lateroventral reticular formation of the medulla oblongata were produced during E11 and E14, mainly on E13. In this region TH-immunoreactive neurons were formed during E10-E11. This discrepancy may be due to the heterogenous population of neurons distributed in the reticular formation of the medulla oblongata. Only several TH-immunoreactive neurons were demonstrated in one section. Thus many TH-non-immunoreactive neurons labeled at a later stage (E12-E14) may be counted in ordinary autoradiographic studies. The phenotype-specific neuronal production sequence is also suggested to occur in the hypothalamic suprachiasmatic nucleus, where VIP-immunoreactive neurons are produced earlier than vasopressin-immunoreactive neurons (34). Another explanation for this discrepancy is the difference in the pattern of production sequence of neurons with the mouse strain, although not yet reported; Taber Pierce used BALB/c mice, and we used ICR mice. Differences in the distribution of catecholamine neurons with the strain have been reported (8).

Few developmental studies using fetal and newborn mouse have been made. Dopamine and noradrenaline have been detected biochemically in the brain of newborn mice although much smaller in their amount than in adult mice (1). No biochemical data have been

reported in fetal mice. Golden (16, 17) revealed catecholamine cell bodies in the midbrain ventral tegmentum on E13 by the Falck-Hillarp histofluorescence technique. These cell groups extend their fibers and arrive at the striatum on E15 (16). Catecholamine-fluorescent cell bodies appear in the dorsal pons on E14 (17). These cells become A6 cells in locus coeruleus of adult mouse. Our findings indicate that neurogenesis of noradrenergic cells occurs before that of dopaminergic cells of the A9 and A10. Considering the present findings and Golden's report, noradrenergic cells were produced earlier than dopaminergic cells but the phenotypic expression of dopaminergic cells was faster than that of noradrenergic cells. Histofluorescence techniques showed that hypothalamic arcuate dopamine neurons first appear on E17 (9). They project to the external layer of the median eminence after birth (9). In this nucleus, phenotypic expression was the slowest among the brain stem catecholamine neuron group. This correlates with the latest neuron production of the A12 cells.

The development of the catecholamine neuron system in other laboratory animals has also been reported (35, 48). Ontogenesis of catecholamine neurons in the rat has been studied by several groups (24, 29, 30, 35, 40, 45, 46). Olson and Seiger (35) reported midbrain dopamine fluorescent cells on E13, fluorescence of noradrenergic neurons on E14 and fluorescence of hypothalamic dopamine neurons on E21. This pattern of development is roughly analogous to that observed in the mouse (9, 17), although fluorescence in the rat appeared slightly earlier in a developmental than comparative stage in the mouse. According to ordinary [^3H]thymidine autoradiographic studies on the neuronal production of each nucleus of the mouse and the rat, neurons in the locus coeruleus are produced mainly on E10 in the mouse (47), and on E12-E14 in the rat (6). Neurons in the substantia nigra are produced on E11-E13 in the mouse (47), and on E13-E15 in the rat (7, 20). Neurons in the hypothalamic arcuate nucleus are produced on E11-E15 in the mouse (41), and on E12-E16 in the rat (3, 25).

By application of catecholamine fluorescence and [^3H]thymidine autoradiography in serial sections, Lauder and Bloom (28) have reported that noradrenergic neurons in the locus coeruleus are produced on E12 and E13, and that dopamine neurons in the substantia

nigra during E13-E15. As in the mouse, noradrenaline neurons in the rat are produced earlier than dopamine neurons; however, their phenotype is expressed in dopamine neurons first.

In contrast to the developmental study using fetal animals by catecholamine fluorescence as a phenotypic marker for catecholamine neurons, Specht *et al.* (45, 46) used a catecholamine-synthetizing enzyme, tyrosine hydroxylase (TH), as a marker. Their study revealed that TH-immunoreactive neurons first appeared simultaneously on E13 in caudal rhombencephalon (in adults, they will be A1, A2), rostral rhombencephalon (A6), ventral mesencephalon (A9, A10), and ventral prosencephalon (perhaps A12). These findings suggest that tyrosine hydroxylase was quickly synthesized within the neurons in the germinal ventricular zone from the matrix cells regardless of whether they are rhombencephalon, mesencephalon, or prosencephalon. Migratory neurons in the mantle layer already synthesize tyrosine hydroxylase but not catecholamine (38, 45). Neurotransmitter catecholamine is thought to be produced after completion of migration (38). The present study revealed that the time lag between the onset of neurogenesis of catecholamine neurons and the onset of appearance of catecholamines is four to seven days in all catecholamine cell groups in mouse brain. Although the developmental feature of tyrosine hydroxylase in the mouse fetus has not yet been studied, the phenotypic expression of a synthetic enzyme 'tyrosine hydroxylase' may be earlier than that of a transmitter 'catecholamine' in mouse catecholamine neurons, considering the sequence of phenotypic expression of rat catecholamine neurons.

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