Transient Coexistence of Tyrosine Hydroxylase and γ -Aminobutyric Acid Immunoreactivities in the Developing Anterior Olfactory Nucleus of the Mouse

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The localization of tyrosine hydroxylase (TH)-immunoreactive (IR) (TH-IR) neurons, which are transiently observed in the anterior olfactory nucleus (AON) of the mouse, was studied. A double-labelling method was used with antibodies against TH, L-3,4-dihydroxyphenylalanine (DOPA), phenylethanolamine-N-methyltransferase (PNMT), γ -aminobutyric acid (GABA), calretinin (CR) and calbindin-D28k (CB). When the total number of TH-IR neurons observed in all regions of the AON obtained from 10- to 14-day-old mice was set at 100%, 30% of TH-IR neurons were observed in the lateral (AOL), 25% in the dorsal (AOD), 20% in the ventral (AOV), 15% in the medial (AOM) and 5% in

both the posterior (AOP) and external (AOE) regions. Approximately 40% of the TH-IR neurons in the AOL to the AOV regions also exhibited GABA immunoreactivity. This corresponds to 20% of the total number of TH-IR neurons observed in all regions of the AON, and also corresponds to 12% of the total number of GABA-IR neurons observed in all regions of the AON. However, TH-IR neurons did not exhibit concomitant PNMT, CR, and CB immunoreactivities. These findings suggest that the TH-IR neurons in the AON of the mouse, a proportion of which is also GABA-IR, may function as a source of neuromodulators for the postnatal growth of GABA-IR neurons.

Key words: Mouse, Anterior olfactory nucleus, Tyrosine hydroxylase, GABA, Colocalization, Immunohistochemistry

I. Introduction

We reported previously that tyrosine hydroxylase (TH)-immunoreactive (IR) neurons are localized in the anterior olfactory nucleus (AON) of a mouse from embryonic day 16 (E16), and that their number reaches, a peak between postnatal day 4 (P4) and 12 (P12). The number of these cells gradually decreases and the cells are no longer observed at postnatal week 4 [23, 24]. We also reported that phenylethanolamine-N-methyltransferase (PNMT)-IR neurons are localized at P10 [23]. TH-IR neurons are neither immunoreactive with antibodies against aromatic L-amino acid decarboxylase (AADC),

the enzyme catalyzing the second step of catecholamine (CA) biosynthesis, nor with antibodies against GTP cyclohydrolase I, the enzyme catalyzing the synthesis of biopterin, a cofactor of TH [25]. In the present study we demonstrate that these TH-IR neurons are not immunoreactive with antibodies against L-3,4-dihydroxyphenylalanine (DOPA). This suggests that the TH-IR neurons found in the AON of the mouse are not involved in CA biosynthesis. We also investigated the immunoreactivity of TH-IR neurons with other neurotransmitters distributed in the AON in order to determine possible functions of the TH-IR neurons. Neurons immunoreactive with CA biosynthesis-related molecules [6, 23, 25] and other neurotransmitters [1, 4, 6, 14, 17, 19, 27–29], as well as receptor-IR neurons [16, 18, 32], are known to exist in the AON. PNMT-, γ -aminobutyric acid-, calretinin- and calbindin-IR neurons are similar to TH-IR

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neurons in terms of morphological characteristics and distribution. Therefore, the colocalization of these other neurochemicals in neurons in the AON was also investigated.

II. Materials and Methods

Fifty male and female mice, ranging in age from postnatal day 10 (P10) to two weeks (P14), were used in the study. The animals were anesthetized with a dose of pentobarbital sodium and perfused with 2% paraformaldehyde + 0.2% glutaraldehyde or 4% paraformaldehyde [10]. Their brains were then removed and immersed in the same fixative at 4°C for 30 min and after washing for 24 hr, 30- μ m-thick serial sections were cut using a cryostat or microslizer (Dosaka EM, Japan). For immunohistochemistry, we used the PAP method or the ABC method [11]. Serial floating sections were incubated in the following primary antisera: rabbit polyclonal anti-TH [21, 22] (1:5,000), (1:5,000): monoclonal mouse anti-TH antibodies (Chemicon, MAB318) (1:1,000): DOPA antiserum [9] (1:2,000): rabbit phenylethanolamine-N-methyltransferase (PNMT) antiserum [20] (1:3,000): rabbit γ -aminobutyric acid (GABA) antiserum [31] (1:5,000): rabbit calretinin (CR) antiserum [30] (1:5,000): and mouse monoclonal calbindin-D 28k (CB) antibodies (Sigma Chemical Co., St. Louis, MO, USA) (1:5,000). In the double-labelling immunofluorescence method, we used mouse antiserum against TH (1:2,000) and rabbit antiserum against GABA (1:3,000), mouse antiserum against TH (1:2,000) and rabbit antiserum against CR (1:2,000), rabbit antiserum against TH (1:5,000) and mouse antiserum against CB (1:3,000), as the primary antisera. The secondary antibodies used for GABA and TH staining were fluorescein-conjugated donkey anti-rabbit immunoglobulin G (IgG) (Chemicon, AP182F) (1:200) and rhodamine-conjugated donkey anti-mouse IgG (Chemicon, AP192R) (1:200). For CR and TH staining, fluorescein-conjugated donkey anti-rabbit IgG (Chemicon, AP182F) (1:200) and rhodamine-conjugated donkey anti-mouse IgG (Chemicon, AP192R) were used, and for TH and CB staining, fluorescein-conjugated donkey anti-mouse IgG (Chemicon, AP 192F) (1:200) and rhodamine-conjugated donkey anti-rabbit IgG (Chemicon, AP182R) (1:200) were used. Stained sections were observed under a confocal laser scanning microscope (Zeiss, LSM 410).

III. Results

Immunohistochemical findings

TH-IR neurons

These were medium-sized (ranging in diameter from 10–20 μ m), circular, oval or spindle-shaped, multipolar or bipolar cells, typically having four to five long, branched varicose processes (Fig. 1A'). When the total number of TH-IR neurons observed in all regions of the AON was set

at 100%, 30% of TH-IR neurons were observed in the lateral (AOL), 25% in the dorsal (AOD), 20% in the ventral (AOV), 15% in the medial (AOM) and 5% in both the posterior (AOP) and the external (AOE) regions (Figs. 1A, 3).

PNMT-IR neurons

These were small- to medium-sized (ranging in diameter from 8–15 μ m), spindle-shaped cells, having relatively straight and unbranched processes with a few varicosities (Fig. 1B'). When the total number of PNMT-IR neurons observed in all regions of the AON was set at 100%, 30% of PNMT-IR neurons were observed in the AOD, and 70% were observed in regions from the AOL to AOV (Figs. 1B, 3).

DOPA-IR neurons

DOPA-IR neurons were not observed in any region of the AON within the distribution range of TH- and PNMT-IR neurons.

GABA-IR neurons

These were medium-sized (ranging in diameter from 10–20 μ m), oval or spindle-shaped cells, having thin and unbranched, short processes without varicosities (Fig. 1C'). When the total number of GABA-IR neurons in all regions of the AON was set at 100%, 40% of GABA-IR neurons were observed in regions from the AOL to AOV, 20% in the AOM, 20% in the AOD and 10% in both the AOP and AOE (Figs. 1C, 3).

CR-IR neurons

These were medium-sized (ranging in diameter from 10–20 μ m), oval or spindle-shaped, bipolar cells, having few branched processes with only a few varicosities (Fig. 1D'). When the total number of CR-IR neurons observed in all regions of the AON was set at 100%, 40% of CR-IR neurons were observed in the AOL, 20% in the AOD, 15% in the AOM, 15% in the AOV and 10% in the AOP (Figs. 1D, 3).

CB-IR neurons

These were medium-sized (ranging in diameter from 10–20 μ m), and most were oval or spindle-shaped cells, having branched processes with few varicosities (Fig. 1E'). When the total number of CB-IR neurons observed in all regions of the AON was set at 100%, 40% of CB-IR neurons were observed in the AOL, 20% in the AOD, 15% in the AOM, 15% in the AOV and 10% in the AOP (Figs. 1E, 3).

Double-immunofluorescence results

TH-GABA colocalization

Colocalization of TH and GABA immunoreactivities was observed in 40% of the TH-IR neurons and 30% of the GABA-IR neurons distributed in regions from the AOL to the AOV (Fig. 2A). This colocalization account-

Fig. 1. Immunohistochemical micrographs showing the AON at P12 after incubation with antisera against TH (A, A'), PNMT (B, B'), GABA (C, C'), CR (D, D') and CB (E, E') (Levels of the Fig. 3E-K). Arrows indicate immunoreactive neurons. A-E $\times 25$; A'-E' $\times 650$.

Fig. 2. Localization of TH, GABA, CR and CB immunofluorescences (Levels of the Fig. 3G–K). **A:** Red (small arrows) indicates TH, green (arrow heads) indicates GABA, and yellow (large arrows) indicates colocalization of TH and GABA immunofluorescences. **B:** Red (small arrows) indicates TH, and green (arrow heads) indicates CR immunofluorescences. **C:** Red (small arrows) indicates TH and green (arrow heads) indicates CB immunofluorescences. **A–C** $\times 300$.

Fig. 3. Schematic drawings of TH (●)-, PNMT (○)-, GABA (■)-, CR (▲)- and CB (△)-immunoreactive neurons from the rostral to caudal regions (A–L) of the AON in frontal sections from postnatal day 12.

Abbreviations: AOB, accessory olfactory bulb; AOD, anterior olfactory nucleus, dorsal; AOE, anterior olfactory nucleus, external; AOL, anterior olfactory nucleus, lateral; AOM, anterior olfactory nucleus, medial; AOP, anterior olfactory nucleus, posterior; AOV, anterior olfactory nucleus, ventral; Den, dorsal endopiriform nucleus; Gl, glomerular layer, olfactory bulb; GIA, glomerular layer, accessory olfactory bulb; Gr, granule layer, olfactory bulb; GrA, granule layer, accessory olfactory bulb; Mi, mitral cell layer, olfactory bulb; Pir, piriform cortex; Tu, olfactory tubercle.

ed for 20% of the total number of TH-IR neurons and 12% of the total number of GABA-IR neurons in the AON.

TH-CR colocalization

Colocalization of TH and CR immunoreactivities was not observed in any of the nuclei of the AON (Fig. 2B).

TH-CB colocalization

Colocalization of TH and CB immunoreactivities was not observed in any of the neurons in the region (Fig. 2C).

TH-PNMT colocalization

In the regions from the AOL to the AOV and the AOD, where the PNMT-IR neurons were distributed, no colocalization of TH and PNMT immunoreactivities was noted.

IV. Discussion

We previously reported that neurons exhibiting immunoreactivity with TH, the rate-limiting enzyme for CA biosynthesis, exist in the AON of the mouse [23, 24]. These neurons were first observed from E16, with their number gradually increasing to reach a peak at P4 to P12, and thereafter, decreasing until TH-IR neurons were no longer observed at around P30 [23, 24]. In addition, in the AON of a mouse, neurons exhibiting immunoreactivity with PNMT, the enzyme responsible for epinephrine biosynthesis, were noted from around P14 until after the mouse reached maturity [24].

In this study, we investigated the relationship between TH-IR neurons in newborn mice aged P10 to P14, during which period the maximal number of transiently existing TH-IR neurons and PNMT-IR neurons were observed. The TH-IR neurons which are transiently existing in the AON of the mouse are immunoreactive neither with CA-biosynthesis-related molecules other than TH or DA [24], nor for DOPA, as observed in this study. Thus, it was confirmed that the TH-containing cells are immunoreactive only for TH and are not involved in the biosynthesis of CA.

TH-IR neurons are also transiently observed in the neocortex [2] and inferior colliculus of the rat [8] and the striatum of the mouse [13]. In these reports, they were assumed to be CA-biosynthesizing neurons. We have previously reported that the transiently observed TH-IR neurons in the raphe nucleus of a newborn laboratory shrew (*Suncus murinus*) also exhibited immunoreactivity with serotonin (5-HT) [11]. In addition, the TH-IR neurons found in the dorsal motor nucleus of the vagus of a laboratory shrew also exhibited immunoreactivity with the acetylcholine (ACh)-biosynthesizing enzyme, choline acetyltransferase (ChAT) [12]. Based on these findings, we suggest that TH functions as a postnatal growth regulator for 5-HT- and ACh-IR neurons.

In the AON of mammals, the existence of neurons IR

with CA-biosynthesis-related molecules [6, 23, 25] and with other various neurotransmitters [4, 14, 17, 27–29], as well as the existence of receptor-IR neurons [16, 18, 32], has been reported. Meanwhile, the colocalization of immunoreactivity with TH and with the calcium-binding proteins, i.e., calretinin (CR) and calbindin-D28k (CB) [7, 26], as well as of TH and GABA immunoreactivities [3, 15] in the various CA-IR neuron groups has been reported. Prompted by these reports, we investigated the possibility of the colocalization of TH immunoreactivity with GABA, CR, CB and PNMT immunoreactivities in the AON of the mouse in terms of cell morphology, using immunohistochemical staining, and in terms of the distribution in various regions, using the immunofluorescence method and confocal laser microscopy. We determined that 20% of the total TH-IR neurons and 12% of the total GABA-IR neurons in the AON showed colocalization of TH and GABA immunoreactivities.

Neurons in the AON are known to excite granule cells by forming synapses with the granule cells of the same side and of the opposite side, and to suppress the activity of mitral cells via dendrodendritic synapses. In this study, we could not confirm the existence of TH- and GABA-IR fibers in the periphery of granular cells. Fibers of GABA-IR neurons in the AON project to the anterior piriform cortex and other regions [5], but fibers exhibiting both TH and GABA immunoreactivities were not observed in these regions. Neurons exhibiting both TH and GABA immunoreactivities observed in regions from the AOL to AOV in this study are considered to function as localized interneurons. The function of TH, which transiently coexists with GABA-IR neurons, remains to be studied; however, it is inferred that TH as a neuromodulator possesses some physiological function other than the biosynthesis of catecholamines. With respect to the large proportion of TH-IR neurons which do not exhibit GABA immunoreactivity, the possibility of the colocalization of TH with other neurotransmitters is being investigated.

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VI. References

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