Postmitotic, Postmigrational Expression of Tyrosine Hydroxylase in Olfactory Bulb Dopaminergic Neurons

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The developmental expression of tyrosine hydroxylase (TOH) was studied in a large, specific population of dopaminergic (DA) neurons in the main olfactory bulb (MOB) of the rat. These DA neurons comprise an anatomically distinctive population that has been well characterized in the adult hamster (Davis and Macrides, 1983) and rat (Halasz et al., 1981; Baker et al., 1983, 1984). We addressed a basic question in developmental neurobiology: What factors regulate the expression of neuronal transmitter phenotype during development? Olfactory bulb DA neurons are born in the ventricular and subependymal zones and migrate through all intervening layers to the most superficial layer in the bulb (Altman, 1969; Bayer, 1983). The time of TOH expression in these neurons was determined using immunohistochemistry and light microscopic image-analysis techniques. The results indicate that TOH phenotype is not expressed when the cells are born in the subependymal zone nor during their migration to the periglomerular region but only after they reached their final destination, the glomerular layer. This suggests that epigenetic factors associated with the glomeruli *initiate* the expression of the key transmitter synthesizing enzyme in these neurons.

Primary olfactory neurons in the nasal epithelium project exclusively to glomeruli of the MOB; removal of this input in *adult* rats (Kawano and Margolis, 1982; Baker et al., 1983, 1984), mice (Nadi et al., 1981; Baker et al., 1983), dogs (Nadi et al., 1981), and hamsters (Kream et al., 1984) appears to down-regulate the expression of the TOH in periglomerular cells. The present results suggested that the input from the primary olfactory nerve is also necessary for the initial expression of the TOH phenotype. In support of this notion, we found that lesions of the olfactory nerve during the first postnatal week caused a significant reduction in the number of TOH-positive juxtglomerular neurons in the following weeks. Thus, the olfactory nerve appears to be necessary for both the *initiation* and *maintenance* of TOH expression in olfactory bulb neurons. These findings suggest that specific cell-cell interactions play a key role in CNS neuronal transmitter phenotype regulation.

The present study also demonstrated that there are significant postnatal changes in the number, size, and distribution patterns of MOB DA neurons. From birth to adulthood there is a linear increase of over 100-fold in the number of neurons expressing the TOH phenotype. There are about 1000 DA neurons per bulb at birth; in adulthood there are approximately 150,000 DA neurons per bulb. The average size of the TOH-immunoreactive cells decreases during development: from $12.1 \pm 0.5 \mu\text{m}$ in the neonate to approximately $10.0 \pm 0.5 \mu\text{m}$ in the adult MOB. This decrease in average size may be related to the fact that there are 2 populations of DA neurons in the adult MOB (Halasz et al., 1981; Baker, 1986; present report). Size histograms of developing and mature MOB DA neurons were generated, and the results suggest that the first MOB neurons to express the dopamine phenotype are probably external tufted cells, which are born earlier than the smaller, more numerous periglomerular neurons which express the phenotype later.

The steady, prolonged increase in the number of neurons expressing the DA phenotype in MOB suggests that these neurons have a progressively important role during the development of olfactory function. This may be related to the steadily increasing ability of rats to discriminate different odors during the first postnatal month (Alberts, 1981).

Determination of the factors that regulate the expression of the neurotransmitter phenotype in developing neurons is a central issue in developmental neurobiology. Most experiments to date have examined neurons in the PNS or in culture. These studies indicate that epigenetic factors such as the integrity of target tissue, presence of afferent inputs, and possibly soluble "trophic" molecules may influence the expression of neurotransmitter phenotype (Furshpan et al., 1976; Reichardt and Patterson, 1977; LeDouarin, 1980; Potter et al., 1981). Thus, it has been hypothesized that epigenetic factors in the PNS influence the initiation and regulation of neurotransmitter phenotype. Much less is known about the factors that regulate the expression of neurotransmitters and peptides in the CNS, doubtless because of the complex connections of central neurons and because they are more difficult to manipulate experimentally than PNS neurons.

One approach to the question of phenotype regulation in the CNS is to determine when and where the transmitter phenotype is initially expressed during development, i.e., is the transmitter expressed at mitosis, during migration, or when the cell inserts into its definitive circuit matrix? Specht et al. (1981a, b) studied developing pros- through rhombencephalic DA neurons in pre-

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natal rats and suggested that some DA neurons express their phenotype during migration. In the brain stem it is difficult to know when migrating DA neurons begin to make and receive contacts. Thus, it is possible that DA neurons that are still migrating have significant interactions with their appropriate pre- and postsynaptic affiliates or have transient contacts with cells that influence the expression of transmitter phenotype. For the DA neurons of the olfactory bulb, it is possible to make a clearer distinction between the mitosis, migration, and contact stages because these neurons are born in the subependymal zone and migrate radially to the most superficial cell layer of the bulb (Shimada, 1966; Altman, 1969), where they establish their definitive synaptic contacts. Additionally, the vast majority of the DA neurons of the olfactory bulb develop postnatally, a circumstance that facilitates their study and the manipulation of their pre- and postsynaptic affiliates. Thus, we know where these neurons are born, their developmental time course, and their route of migration. It has also been demonstrated that the dopamine (DA) levels and tyrosine hydroxylase (TOH) activity in the *adult* bulb are influenced by epigenetic factors (Nadi et al., 1981; Kawano and Margolis, 1982; Brunjes et al., 1985). TOH immunoreactivity in juxtglomerular neurons (comprising periglomerular and external tufted cells) in the adult bulb is severely reduced or eliminated subsequent to removal of primary olfactory nerve (PON) input to the olfactory bulb glomeruli (Baker et al., 1983, 1984; Kream et al., 1984). Thus, the olfactory nerve has a regulatory action on TOH activity in the adult bulb. It is possible, therefore, that the developmental induction of TOH expression is also influenced by olfactory nerve input. In the present study, we lesioned the olfactory nerve in developing animals to assess its role in the *initiation* on the TOH phenotype.

The purposes of this study, therefore, were to specify the postnatal development of DA neurons in main olfactory bulb (MOB) using immunohistochemistry and lesions of the olfactory nerve to determine if the TOH phenotype is expressed at *mitosis*, during *migration*, or *inserted* (Fig. 1). The central hypothesis is that induction of the TOH phenotype depends upon specific postmitotic, postmigrational cell-cell interactions.

Materials and Methods

Animal and tissue preparation. Sprague-Dawley rats aged 1, 2, 3, 4, 5, 8, 10, 11, 14, 16, 22, 28, 32, 37, 50, 60, and 64 d or older adults were used in this study. Postnatal day 1 (P1) is defined as the day of birth, while adults are considered P60 or older. Two to five rats were used at each age studied. The rats were anesthetized deeply with sodium pentobarbital and perfused transcardially with saline (room temperature) for 2 min. The saline rinse was followed by perfusion with 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min (500 ml). Brains were removed from the skull, left in the fixative for at least 1 hr, and placed in phosphate-buffered 20% sucrose overnight.

Immunohistochemistry. Thirty-micron-thick frozen sections of the olfactory bulb were cut in the transverse plane and placed in 0.1 M PBS at pH 7.4. Free-floating sections were processed for immunohistochemistry using the following protocol: Sections were (1) rinsed in 0.1 M PBS, (2) incubated in primary antiserum or without primary antibody in 0.1% Triton X-100, 2% normal goat serum (NGS) in PBS for 15–40 hr in the cold on a shaker table, (3) rinsed 2×10 min in PBS, (4) incubated in goat anti-rabbit IgG (Cappel) diluted 1:100 in the same diluent as in step 1 for 1 hr at room temperature, (5) rinsed 2×10 min in PBS, and (6) incubated 1 hr in rabbit peroxidase-antiperoxidase (Dakopack, Accurate Chemicals, Inc.) diluted 1:100 in same diluent as in step 1. Procedures 3–6 were sometimes repeated. After 2×10 min rinses in PBS, sections were incubated for 10–20 min in a PBS containing 0.05% diaminobenzidine dihydrochloride and 0.01% H_2O_2 . Sections were then

Figure 1. Hypothesis of when neurons in MOB could begin to express the TOH enzyme during development. EPL, external plexiform layer; EZ, ependymal/subependymal zone; GCL, granule cell layer; GL, glomerular layer; IPL, internal plexiform layer; MCL, mitral cell layer.

rinsed 2×10 min in phosphate buffer, mounted on subbed slides, rinsed in distilled water, dehydrated, and coverslipped.

The primary antibody was polyclonal rabbit anti-TOH diluted 1:500–1:1000. The antibody was a generous gift from Dr. Tom Finger and has been characterized previously for specificity (Morita and Finger, 1987). The TOH immunoreactivity is specific for DA neurons but may also detect neurons containing catecholamines other than dopamine. Thus, some sections of MOB were incubated with an antibody to dopamine-beta-hydroxylase, a requisite enzyme for noradrenergic (NE) neurons or with antibodies to PNMT to detect potential epinephrine (E) neurons. No NE or E neurons were detected in MOB at any of the ages studied. Thus, the anti-TOH appears to label only DA neurons in MOB. Additional evidence that the TOH-immunoreactive (IR) staining is a definitive marker for MOB DA cells is provided by biochemical studies showing that TOH is closely associated with DA levels in the olfactory bulb glomerular layer (Nadi et al., 1981).

Nissl-stained sections. The location and distribution of TOH-IR neurons at each developmental age were correlated with the developing cytoarchitecture of MOB in sections that were cut serially in the transverse plane at $40 \mu\text{m}$ and stained with cresyl violet. In addition, some sections that had been processed for TOH were counterstained with cresyl violet for Nissl so that the relative abundance of TOH-IR cells versus non-TOH-IR periglomerular cells at different stages of development could be assessed directly.

Olfactory nerve lesions. Five rat pups at P3–7 were anesthetized with 0.02 ml of a mixture of 9 parts ketamine (40 mg/kg) and 1 part acepromazine (0.2 mg/kg). The cartilage dorsal to the nasal epithelium was removed on one side, the epithelium was exposed by a parasagittal incision, and a gelfoam pledget containing 1–2 μl of 0.17 M $ZnSO_4$ was placed in the nasal cavity. A similar procedure (nasal irrigation with $ZnSO_4$) effectively and permanently destroys the PONs whose axons comprise the olfactory nerve (rat: Margolis et al., 1974; hamster: Kream et al., 1984). Following surgery, the pups were kept warm until they were ambulatory. They were then returned to their mother until the time of sacrifice or weaning. All rats were housed and maintained in AAALAC approved facilities in the Department of Laboratory Animal Medicine at the University of Cincinnati. After a 7–14 d survival, the animals were sacrificed, and their brains were processed for immunohistochemistry as described above.

Image analysis. Sections of the rat olfactory bulb were examined under bright-field optics and imaged with a Newvicon TV camera through a digital real-time video processor (Hughes Aircraft Co., Image Model

Figure 2. A–F, Bright-field photomicrographs of Nissl-stained (left photos) and TOH-IR (right photos) transverse sections obtained at different postnatal ages. The intensity of TOH-IR staining in cells is relatively consistent across different ages. Scale bar, 100 μ m. For abbreviations, see Figure 1.

794) and fed to a Magiscan 2A (Joyce-Loebl) image-analysis system. The labeled neurons were analyzed throughout the thickness of the section. The image-analysis system displays live images on a 512 $\times$ 512 high-resolution monitor. Reconstruction of live images of 1024 $\times$ 1024 pixels and greater has been made possible by a program that controls movements of the microscope stage and focus control. Thus, the Magiscan system is used to identify, precisely plot, and measure all labeled neurons in relation to selected anatomical landmarks; cytoarchitectonic boundaries are also plotted. The area, perimeter, length, and breadth

of labeled neurons were measured, and statistical analysis of the results was performed using the Student *t* test to determine if there were differences in juxtglomerular DA neurons in different parts of the glomerular region and/or at different developmental ages.

Results

The postnatal development of DA neurons in MOB is detailed below. Because the developmental patterns and numbers of

TOH-IR cells are similar at some developmental ages, results from these ages were grouped for purposes of description. Most of the results and illustrations are based on observations of mid-rostrocaudal levels of MOB unless otherwise stated, although all rostrocaudal levels of MOB were analyzed. To correlate the locations of TOH-IR neurons with the cytological development of the olfactory bulb, brief descriptions of Nissl-stained sections of MOB at different stages of development are presented for each age group first, followed by an account of the TOH-IR neurons.

P1–8 stage

Nissl staining. In Nissl-stained sections of the MOB, only a few glomeruli of P1 rats are visible, and they are very small (Fig. 2A). These glomeruli are up to 35 μm in diameter and are surrounded by many small darkly stained presumptive neurons and/or glia. By P2, more glomeruli are visible, and some are as large as 50 μm in diameter. The external plexiform layer is about 50 μm thick and contains numerous small neurons (Fig. 2C). By the end of the first postnatal week, most glomeruli are visible,

and the largest of them have diameters of 70–80 μm . The external plexiform layer is about 75–100 μm thick. The number of neurons in the external plexiform layer increases substantially around P4 (Fig. 2E). This increase may be the result of migrating juxtglomerular neurons since a large proportion of these interneurons are born during this period (Bayer, 1983) and are probably en route from their birthplace in the ependymal zone to their final destination in the glomerular layer. By P8, the number of neurons surrounding the glomeruli or between glomeruli has increased substantially so that they are packed about 10 deep between glomeruli or between glomeruli and the external plexiform layer (Fig. 2G).

TOH immunoreactivity. From P1 through P8, TOH-IR neurons are scattered around the circumference of the bulb and are generally located on the deep aspect of glomeruli (Figs. 2, 3). No TOH-IR neurons were visible in the mitral cell layer or deeper layers of the bulb. Most of the TOH-IR cells were between 11.5–13 μm in long axis (Table 1). At P1, the dendrites of individual labeled neurons are well developed and often ramify within a single glomerulus (Fig. 2B). At this early age, these dendritic ramifications are readily visualized because typically only one neuron is associated with each glomerular profile; in a few instances the dendrites enter more than one glomerulus. During the latter part of the first postnatal week and at subsequent ages, the number of DA neurons/glomerulus increases

rapidly, and it becomes progressively more difficult to follow dendrites belonging to specific neurons and, thus, to determine what proportion of the neurons extend their dendrites into more than one glomerulus.

P14–22 stage

Nissl staining. The glomeruli of rats in the third week are substantially larger than those of rats in the first week of postnatal life. Glomerular diameters range from 50 to 125 μm . There are fewer neurons intervening between glomeruli by this time: 5–6 compared with 7–10 at P8. The decrease in the packing of interglomerular neurons may reflect the reduced rate of periglomerular cell birth and migration of neurons by this developmental age (Bayer, 1983), which would cause the thinning out of cells as they relocate to surround the growing glomeruli; alternatively, it could represent cell death. By this time the external plexiform layer has expanded to up to 200 μm in thickness and contains cells of many sizes.

TOH immunoreactivity. Many more TOH-IR neurons are present during this period than at P1–8 (Figs. 2 and 3). The neurons are predominantly located in the deeper portions and to a lesser extent around the remaining circumference of the glomeruli. The range of cell sizes at this age is from 6 to 20 μm , with the mean around 11.5 μm (Table 1). On average, 5 neurons are associated with individual glomerular profiles in 30- μm -

Figure 3. Computer-assisted reconstruction of transverse sections through the MOB showing the distribution of TOH-IR neurons during development. The labeled neurons are noted by dots. Scale bar, 1 mm. For abbreviations, see Figure 1.

thick transverse sections, although some glomerular profiles have more and some still lack DA neurons. There was no observable systematic relationship between the size or location of glomeruli with DA neurons and those lacking DA cells at those ages.

P32–40 stage

Nissl staining. The olfactory bulb continues to increase in size during this period, and the size of glomeruli also increases to up to 150–200 μm in diameter. Juxtaglomerular cells are packed 3–4 deep between glomeruli by this stage.

TOH immunoreactivity. During this 32–40 d stage, the distribution of TOH-IR neurons begin to look much like that found in the adult. The average size of the juxtaglomerular TOH-IR cells has decreased compared with earlier stages of development (Table 1). The main differences between the TOH-IR neuron distribution at P37 and in adults are (1) the size of the glomeruli, which is slightly larger in the adult, and (2) the number of TOH-IR neurons observed in each section, which is greater in the adult (Table 2). Basically, however, the pattern of DA juxtaglomerular neurons is similar to adult patterns by the fifth postnatal week.

Adult stage (>P60)

Nissl staining. The glomeruli in the adult MOB range from 100 to 160 μm in diameter. The glomeruli are surrounded by small cells packed 1–2 layers thick except in interglomerular regions, and the deep aspect of glomeruli where multiple layers of small neurons occupy the neuropil (Fig. 2O). The glomeruli on both

Table 1. Average size of DA juxtaglomerular cells during development

Postnatal age (d)	No. of rats	Total neurons measured (n)	Length (μm , mean $\pm$ SD)
1	5	86	12.1 $\pm$ 0.5
4	2	67	12.5 $\pm$ 0.1
8	5	187	12.4 $\pm$ 0.2
15	2	184	11.5 $\pm$ 0.4
37–43	3	671	9.8 $\pm$ 0.2
>60	4	292	10.0 $\pm$ 0.5

Figure 4. Bright-field photomicrographs of olfactory bulb sections stained to visualize TOH-IR neurons in a P15 rat, one week after the olfactory nerve was lesioned using ZnSO_4 . *A*, Contralateral to the lesion; *B*, ipsilateral to the lesion.

the medial and lateral sides of the bulb are significantly larger than those located ventrally, dorsally, and at the rostral pole of the bulb.

TOH immunoreactivity. Immunocytochemical expression of TOH is most prevalent in periglomerular and external tufted neurons located around glomeruli in the main olfactory bulb. Collectively these cells are named juxtaglomerular cells. TOH-IR staining is infrequently present in middle tufted cells and only rarely in cells located in the mitral cell or granule cell layer. This corresponds very well to previous descriptions of the adult rat (Halasz et al., 1977, 1981; Baker et al., 1983; Seroogy et al., 1985) and hamster (Davis and Macrides, 1983). The most striking feature of the staining is that the TOH-IR cells form a ring around glomeruli, to the extent that their presence helps demarcate glomeruli. On average, 16–17 neurons surround each cross-sectional glomerular profile in the adult. The dendrites of these juxtaglomerular DA neurons ramify almost exclusively within the glomeruli.

Olfactory nerve lesions

Following ZnSO_4 lesions of the nasal epithelium in P3–7 animals, the olfactory bulb ipsilateral to the lesion contained very few TOH-IR neurons compared with the contralateral bulb (Fig. 4) and littermate controls. We did not determine if the olfactory nerve lesions were complete. However, the ipsilateral nerve

layer and olfactory bulb were greatly reduced in size, indicating successful total or partial destruction of the epithelium. Some faint TOH immunoreactivity could still be observed in many ipsilateral juxtaglomerular neurons 1 week after the operation, but very few neurons had TOH immunoreactivity equaling that found on the contralateral side. We conclude that if the olfactory nerve does not reach the glomeruli during development, then the DA neurons fail to express the TOH phenotype. Therefore, the olfactory nerve is important for the initial expression of TOH. We have not determined whether the olfactory nerve-deprived juxtaglomerular neurons remain viable without ever getting the PON input.

Development of olfactory bulb DA neurons

Certain trends were observed in the development of neurons expressing TOH activity in the MOB.

Cell number. The most salient observation is that at all ages, from neonate to adult, TOH-IR neurons in MOB are associated with the glomerular layer. Another obvious trend is that the number of TOH-IR neurons increases steadily from birth to adulthood. In 30- μm -thick frozen sections taken from middle levels of MOB, the DA cell numbers increase linearly ($R = 0.945$) at a rate of approximately 17 cells/d at least until around P60 and then the numbers remain relatively constant during young adulthood (Fig. 5; aged animals have not yet been analyzed). Thus, there is a linear increase in the number of neurons expressing TOH per section throughout development. There was no indication of periods of unusually high or low rates of increase in TOH expression at any time during development, nor were there any obvious systematic regional differences in TOH expression: neither medial to lateral, dorsal to ventral (Fig. 3) nor rostral to caudal gradients in the expression of the dopamine phenotype were observed. Therefore, it appears that the TOH-IR neurons begin to express the TOH phenotype uniformly in glomeruli in all regions of MOB.

The total number of TOH-IR neurons in MOB at different developmental ages was estimated by calculating the average size of olfactory bulbs and average number of TOH-IR cells per section at each stage. From birth to adulthood we estimate that the increase in TOH-IR neurons in each olfactory bulb is 100- to 150-fold from approximately 1000 at birth to 150,000 in the

Table 2. Estimates of the number of TOH-IR neurons in the MOB during development

Age (d)	Approximate length of olfactory bulb (mm)	Number of 30 μm sections/olfactory bulb	Number of TOH-IR cells/30 μm section	Estimated number of TOH-IR neurons/olfactory bulb
1–2	1.25	40	20–60	800–2400
8	2.0	70	50–100	3500–7000
14–15	2.3	75	130–250	10,000–18,000
22	2.5	80	450–500	35,000–40,000
37–40	3.5	120	650–800	75,000–100,000
>60	4.0	130	750–1110	100,000–150,000

adult (Table 2). The average number of TOH-IR neurons per glomerular profile also increases substantially. Generally, younger rats, P1–2 have 0–3 neurons associated with a glomerular profile. P4 rats have 1–5 neurons/glomerular profile, P8 rats have 3–7 neurons, P14 rats have 4–12 neurons, P28 rats have 7–17 neurons, while P37 rats and adults have 14–30 neurons surrounding each glomerulus in a given profile. The glomeruli are somewhat difficult to differentiate in the first week, but it is clear that they do increase in size from birth to adulthood. Interestingly, by the second week, glomeruli can be unambiguously identified in Nissl material, and there are approximately 55–90 glomerular profiles per section taken from the largest part of the MOB; this range remains relatively constant through subsequent developmental stages to adulthood. Thus, at least from week 2, the number of glomeruli in each section does not increase with age, but the average size of glomeruli and the olfactory bulb continue to increase until at least P60.

Cell types. Previous studies have suggested that there are 2 populations of DA neurons in the adult MOB, based on size or location (Halasz et al., 1981; Baker, 1986). Based on size histograms alone, we could not detect subpopulations within the adult juxtglomerular DA population, i.e., the size–frequency distribution of DA neurons in adult bulbs was unimodal (Fig. 6B). However, when location was taken into account, we found significant differences in the adult DA population: DA neurons located in the external plexiform layer were significantly larger ($\bar{x} = 12.1 \pm 0.7 \mu\text{m}$, $p < 0.05$) than those with a juxtglomerular location ($\bar{x} = 10.0 \pm 0.5 \mu\text{m}$). Furthermore, juxtglomerular DA neurons located on the deep half of glomeruli were significantly larger ($\bar{x} = 10.6 \pm 0.8 \mu\text{m}$, $p < 0.05$) than those ($\bar{x} = 9.3 \pm 0.4 \mu\text{m}$) located around the superficial half of glomeruli.

There is a gradual decrease in the average size of DA neurons after the first postnatal week (Table 1). The decrease in the size of the neurons was significant ($t = 5.6$, $p < 0.05$) when P8 neurons ($n = 5$ rats) were compared with adult neurons ($n = 5$). Size–frequency histograms of P8 and adult juxtglomerular DA neurons showed unimodal bell-shaped curves for each population. However, there was a shift in the average sizes of the neurons such that over 70% of the DA neurons at P8 were larger than $11 \mu\text{m}$, while in the adult only about 20% of the juxtglomerular DA neurons were larger than $11 \mu\text{m}$ (Fig. 6A–C). Although the proportion of larger DA neurons decreases in the adult bulb compared with the neonate, there are still many of the larger neurons present, particularly in deep juxtglomerular or external plexiform layer positions. Thus, there appears to be a change in the DA neuronal population with development. As will be argued below, this change probably reflects the later development of smaller, more superficial DA neurons.

Discussion

Developmental expression of DA neurons in MOB

During development, olfactory bulb neurons destined to express the dopamine phenotype are born in the ventricular or subependymal zone and migrate to their destinations in the most superficial cell layers of the bulb (Hinds, 1968; Altman, 1969; Bayer, 1983; Kishi, 1987). The major finding of the present study was that DA neurons never express the TOH phenotype at the time of birth or during their migration from the proliferative zone through the deep layers of the bulb, but only after they have reached their destination in or immediately subjacent to the glomerular layer. Thus, the transmitter phenotype of this population of CNS neurons appears to be developmentally reg-

Figure 5. Graph showing an estimate of the number of DA neurons in 30- μm -thick transverse sections during development. Each point represents the number of neurons observed in individual sections taken through a middle rostrocaudal level of MOB. The line represents the slope of a linear regression ($R = 0.945$) of the increase in neurons between birth and P64.

ulated by postmitotic, postmigrational events. We hypothesize that these interneurons only express the TOH phenotype after they insert into their definitive circuit matrix. Thus, it is likely that the trigger for TOH expression in MOB is some factor spatially restricted to the glomerular layer.

Such a developmental sequence for TOH expression may not be universal for CNS neurons. For example, many neurons in the pros-, mes-, and rhombencephalon express TOH immunoreactivity when they are still migrating (Specht et al., 1981a). It might be argued, therefore, that TOH expression in these neurons is independent of target-afferent interactions since expression is observed during their migration. The difficulty with this interpretation is that it is not known whether brain-stem DA neurons have contacts with their eventual targets and/or receive synaptic inputs from their definitive afferents while they are still migrating. In the case of the olfactory bulb it is conceivable that migrating pre-DA neurons have made contacts, but it is most likely that these interneurons do not establish adultlike circuit connections until they reach the glomeruli. Thus, the apparent discrepancy between brain-stem neurons that express TOH during migration and MOB neurons whose TOH expression is postmigratory may not be an essential difference because brain-stem neurons may make and/or receive contacts during migration. The key issue is whether the transmitter phenotype is triggered by specific cell–cell interactions in the glomeruli, and further studies, including EM analysis, are needed.

The triggering of the dopamine phenotype in the juxtglomerular region may indicate that the neurons require an input from the primary olfactory neurons since the olfactory nerve terminates heavily and exclusively in the glomeruli. Indeed, in the MOB of the adult rat, the maintenance of DA synthesis in periglomerular cells depends upon integrity of primary olfactory afferent input to MOB (Nadi et al., 1981; Baker et al., 1983, 1984). Thus, when the PON input to MOB is removed, TOH expression is reduced or abolished in juxtglomerular cells (Baker et al., 1983, 1984). However, the loss of the PON input does not seem to affect the viability of the juxtglomerular cells, and many appear to regain their DA phenotype when PON axons regenerate into the glomeruli (Baker et al., 1983, 1984).

Antibodies to olfactory marker protein have been used in

Figure 6. Frequency histograms showing the size distribution of DA neurons in (*A*) PND 8 MOB, (*B*) adult juxtglomerular neurons, (*C*) PND 8 and adult compared, and (*D*) neurons in the adult external plexiform layer (EPL). The histograms were generated from the total number of neurons in all animals observed (2–3 sections/animal, 4–5 animals/histogram).

studies as markers for maturity and, hence, synaptic connection between primary olfactory neurons and the olfactory bulb in the rat (Farbman and Margolis, 1980). These studies indicated that the first connections between epithelium and olfactory bulb are made 1–3 d before birth, and the intensity and staining patterns continue to increase until 1 month after birth. Recent studies confirm through electron microscopic observation that the first synapses form in the glomerular layer at embryonic day 18 (A. Farbman, personal communication). The progressive developmental increase in the PON connections with the olfactory bulb thus parallels the increase in TOH-IR neurons around glomeruli during development. Therefore, PON axons appear to be important for the developmental *induction* of the TOH phenotype in juxtglomerular cells. Our nasal ZnSO_4 lesions drastically reduced the PON inputs to the developing MOB and reduced TOH expression in juxtglomerular cells, further suggesting that the PON input is necessary (but not necessarily sufficient) for the initiation of TOH expression.

Another indication of the importance of PON input for the initiation of DA expression is the temporal relationship between neuron birth and the expression. The expression of TOH phenotype lags somewhat behind the birth curve of periglomerular neurons (Bayer, 1983). For example, most periglomerular neurons are born by P14, the majority being generated in or prior to the first postnatal week (Bayer, 1983). A few TOH-IR neurons are present in the MOB before birth (Specht et al., 1981b), but most DA neurons in the MOB do not express the TOH phenotype until after birth, and it is a gradual steady increase that does not peak until about the 7th postnatal week. Thus, since

most periglomerular cells are born in the first 2 postnatal weeks (Bayer, 1983), most of them are probably migrating or may already be in the glomeruli several weeks before they begin to express immunocytochemically detectable levels of TOH. The gradual increase in TOH-IR cells, thus, appears to correlate well with glomerular maturation and gradual increase in PON inputs to MOB during development (Farbman and Margolis, 1980) and not as much with time of birth or migration of the periglomerular neurons.

Mechanism of TOH expression

The foregoing discussion strongly suggests that the initial TOH expression *requires* olfactory nerve input. However, it is not known if the regulatory effect of the olfactory nerve on TOH expression is mediated via pre- or postsynaptic mechanisms or both. In the PNS, both pre- and postsynaptic regulation of TOH expression have been reported (Black, 1978; Katz and Black, 1986); preganglionic axons in the sympathetic system have regulatory effects on postsynaptic postganglionic TOH-IR cells (Black, 1978), while TOH expression in pseudounipolar neurons in the petrosal ganglion appears to be influenced by their peripheral target, the carotid body (Katz and Black, 1986). In the olfactory bulb, it is not known whether PON axons terminate directly on the juxtglomerular DA neuron. Electron microscopic studies indicate that PON terminals contact the dendrites of periglomerular, external tufted cells and mitral cells (Shepherd, 1972). Thus, it is possible that TOH expression is triggered and maintained by PON contacts on DA neurons. On the other hand, periglomerular and tufted cells have been reported to

express neurotransmitters and peptides other than dopamine (Seroogy et al., 1985; Baker, 1986; Gall et al., 1986). At least 2 laboratories have reported that some periglomerular neurons coexpress DA and GABA in the adult rat (Kosaka et al., 1985, 1987b; Gall et al., 1987) and during development (Kosaka et al., 1987a). However, there have been no EM immunocytochemical studies of the relations between chemically identified neurons and synapses in the glomerular layer. Thus, while PONs may synapse directly on DA neurons, it is also possible that PONs contact non-DA periglomerular neurons and, thus, influence TOH expression via an action on periglomerular neurons that are the *targets* of periglomerular DA neurons. A third possibility is that PONs contact centrifugal afferent terminals that are presynaptic to DA periglomerular neurons. We have recently demonstrated a dense serotonergic input to the glomeruli from the raphe nuclei (McLean and Shipley, 1987a, b) and heavy cholinergic input from the basal forebrain (Shipley et al., 1986; and in preparation). It is not known whether these centrifugal fibers terminate on DA neurons nor whether PON axons are presynaptic to centrifugal terminals. Thus, while there is good evidence that the olfactory nerve is necessary for the initiation (this report) and maintenance (Kawano and Margolis, 1982; Baker et al., 1983, 1984) of TOH expression in MOB DA neurons, it is unclear whether this regulatory action is due to a *direct* action of PON terminals on DA neurons or *indirect* actions mediated via PON contacts upon postsynaptic targets of DA neurons or on afferents presynaptic to the DA neurons.

The best studied example of synaptic regulation of TOH is in the PNS (Black and Geen, 1974), in which it has been shown that cholinergic synapses acting on nicotinic receptors regulate postsynaptic TOH. A similar mechanism might operate in the olfactory bulb, as we have recently shown that there are cholinergic inputs to the glomerular layer (Shipley et al., 1986; and in preparation) and the glomerular layer contains dense binding of nicotinic, but not muscarinic, receptor ligands (Blaha et al., 1984). Further study is necessary to determine whether this nicotinic synapse interacts with the DA neurons and/or the olfactory nerve input.

Sensitivity of TOH staining

A potential objection to our interpretation that TOH expression is triggered by some factor relating to the glomerular layer is that our immunohistochemical procedure may not be sensitive enough to detect extremely low levels of TOH in the tissue sections. Although lack of sensitivity is possible, changes in levels of TOH-IR neuron expression appears to correlate well with published biochemical measures of TOH and dopamine activity in the MOB (Nadi et al., 1981). Second, if migrating neurons have low levels of TOH, then the levels in at least some of them should have been detected by immunohistochemistry. Unless the levels of the enzyme are very tightly regulated in every neuron, the sheer magnitude of the population would lead one to expect that at least a small percentage of the cells would have immunocytochemically detectable levels of TOH during their migration. TOH expression in migrating neurons was never observed, leading us to suggest that the phenotype is not triggered until the neurons have reached their destination. Even if low levels of TOH are present in migrating neurons, it still appears that events associated with the neurons reaching the glomerular layer must trigger a large increase in TOH expression because lesions of the epithelium during the initial postnatal week cause a drastic reduction in the number of TOH-positive

neurons observed in the second postnatal week. Thus, we hypothesize that expression occurs only after the neurons have reached their target layer. Clearly, it will be important to pursue this issue further. One way to do this may be to determine whether TOH RNA expression is initiated when the neurons reach the glomeruli. *In situ* hybridization techniques using probes for TOH mRNA might shed additional light on this question.

Position and size relationships of periglomerular cells

TOH-IR neuron sizes and their juxtglomerular distribution change during development. We observed that most of the TOH-IR neurons are located on the deep portion of the glomeruli in early postnatal development, whereas by adulthood the neurons surround the glomeruli. However, we were unable to determine if the TOH-IR neurons that are observed around the deep portion of the glomeruli in the neonate migrate to more superficial juxtglomerular positions or whether they remain set in the deep position through adulthood and comprise the later-arriving superficially located cells. TOH-IR neurons in the neonate were generally larger than the average size of TOH-IR juxtglomerular neurons at later stages of development. In addition, we observed that in older animals deeply placed DA neurons are larger than more superficially located DA neurons. It is possible, therefore, that the first juxtglomerular neurons in the MOB to express TOH activity are the larger, more deeply located external tufted cells. In such a scheme, as development continues, smaller, more superficially located and later-born periglomerular neurons would begin to express the DA phenotype; the increasing number of smaller neurons would account for the average decrease in size of TOH-IR neurons that surround glomeruli.

Function of DA neurons in the developing MOB

We have estimated that there are well over 100,000 DA neurons in MOB of the adult rat. The biological significance of these DA neurons is unknown, but their sheer number and the fact that they are positioned at the first level of synaptic integration in the olfactory system argues that their importance to physiological processing in MOB must be substantial. The role of DA neurons in development is also unclear. However, the gradual increase in the number of neurons that express TOH-IR in the bulb may correlate with the animal's gradual ability to detect and discriminate odors (Alberts, 1976, 1981).

In some other parts of the brain, including the di-, mes-, and rhombencephalon, TOH-IR cells appear very early in development and have been proposed to have a trophic role (Specht et al., 1981a). However, in the MOB, TOH expression is relatively late in development, and, therefore, it is unlikely that these cells have significant trophic or inductive roles in the bulb. On the other hand, these DA neurons may play a role in synaptic stabilization. DA has been shown to inhibit growth cones in retinal neurons (Lankford et al., 1986). If a similar mechanism operates in the bulb, DA neurons may function to stabilize developing and replacement PON synapses, as there is some turnover of PONs and their glomerular synapses throughout the life of the animal (Graziadei and Monti Graziadei, 1978a, b).

In summary, we have shown that there is a steady increase in the number of neurons expressing TOH-IR in the developing rat olfactory bulb. The olfactory nerve is necessary for the *initiation* (present study) and maintenance (Margolis et al., 1974; Nadi et al., 1981; Baker et al., 1983, 1984; Kream et al., 1984) of the TOH expression in MOB DA neurons, suggesting that

specific cell-cell interactions underlie the regulation of this neuron's transmitter phenotype. Whether epigenetic factors such as cell-cell interactions are fundamental to the induction or maintenance of transmitter phenotype in other central neuronal populations is not known, but the potential for the principles operating for olfactory bulb DA neurons to be a general phenomena is intriguing. The present study provides a chemoanatomical framework for further investigating the specific developmental events that regulate TOH expression in one well-defined population of CNS DA neurons and provides an example of postmitotic, postmigrational induction of a CNS transmitter phenotype that may be common to other transmitter systems.

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