Phenotypic Differentiation during Migration of Dopaminergic Progenitor Cells to the Olfactory Bulb

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A possible source for transplantable neurons in Parkinson’s disease are adult olfactory bulb (OB) dopamine (DA) progenitors that originate in the anterior subventricular zone and reach the OB through the rostral migratory stream. We used adult transgenic mice expressing a lacZ reporter directed by an 8.9 kb tyrosine hydroxylase (TH) promoter to investigate the course of DAergic differentiation. Parallel transgene and intrinsic TH mRNA expression occurred during migration of DA interneurons through the mitral and superficial granule cell layers before these cells reached their final periglomerular position. Differential transgene and calcium–calmodulin-dependent protein kinase IV expression distinguished two nonoverlapping populations of interneurons. Transgenic mice carrying a TH8.9kb/lacZ construct with a mutant AP-1 site demonstrated that this element confers OB DA-specific TH gene regulation. These results indicate that DA phenotypic determination is specific to a subset of mobile OB progenitors.

Key words: tyrosine hydroxylase; Parkinson’s disease; stem cell; migration; rostral migratory stream; subventricular zone; dopamine

Delineating the mechanisms underlying development of dopaminergic (DAergic) systems is the focus of numerous studies because of its relevance to understanding the etiology of and developing restorative therapy for Parkinson’s disease. The laminar organization and primarily postnatal development of the olfactory bulb (OB) make it an ideal model system for studying DA phenotypic differentiation (Hinds, 1968a,b; McLean and Shipley, 1988; Baker and Farbman, 1993). Significantly, OB interneurons, including periglomerular DA cells, are produced even in adults (Luskin, 1993; Doetsch and Alvarez-Buylla, 1996; Lois et al., 1996; Suhonen et al., 1996; Alvarez-Buylla and Temple, 1998; Doetsch et al., 1999a) thus providing an autologous source for transplantable DA neurons.

Adult OB DA neurons derive from stem cells in the anterior subventricular zone (SVZa), a remnant of the lateral ganglionic eminence that also contributes interneurons to the cortex (Anderson et al., 1999). Dlx1/2-deficient mice had reduced numbers of granule cells in the cortex as well as the OB (Anderson et al., 1999). Dlx1/2-deficient mice had reduced numbers of granule cells in the cortex as well as the OB (Anderson et al., 1999). SVZa-derived progenitor cells migrate through the RMS to populate granule and periglomerular layers of OB (Betarbet et al., 1996). DA markers including protein and mRNA for tyrosine hydroxylase (TH), the first enzyme in DA biosynthesis, were reported previously only in the periglomerular region of the OB (Stone et al., 1991; Baker and Farbman, 1993; Betarbet et al., 1996; Suhonen et al., 1996).

None of the above studies addressed the issue of when and where OB progenitor cells attain the capacity to differentiate into DA neurons. This question was examined in transgenic mice produced in our laboratory that exhibit high-level tissue-specific expression of a construct with 8.9 kb of TH promoter driving a lacZ reporter gene (Min et al., 1994). The findings suggested the existence of two populations of granule cells, only one of which is capable of differentiating into DA neurons.

**MATERIALS AND METHODS**

**Animals and surgery.** All procedures were performed under protocols approved by the Institutional Animal Care and Use Committee of Cornell University and conformed to National Institutes of Health Guidelines. Adult transgenic TH8.9kb/lacZ mice were produced in our laboratory as described previously (Min et al., 1994). These mice were one of the six lines derived that exhibited high-level lacZ expression that is specific to catecholamine-expressing neurons (Min et al., 1994). The production of the mice with the mutant AP-1 site is described below. Naris closure was performed as described previously under pentobarbital
anesthesia (30 mg/kg) using a bipolar coagulator (Liu et al., 1999). Mice were killed at least 2 months after closure. All animals were housed under a 12 hr light/dark cycle with food and water ad libitum.

For immunohistochemical, histochemical, and in situ hybridization procedures, mice were anesthetized with an overdose of pentobarbital (90 mg/kg). They were then perfused transcardially with saline containing 0.5% sodium nitrite and 10 U/ml of heparin followed by either saline or 10% formaldehyde (for immunocytochemistry and in situ hybridization) or 2.5% glutaraldehyde and 0.5% formaldehyde (for immunocytochemistry and in situ hybridization) or 2.5% glutaraldehyde and 0.5% formaldehyde (for immunocytochemistry and in situ hybridization) or 2.5% glutaraldehyde and 0.5% formaldehyde (for immunocytochemistry and in situ hybridization).

For double-label immunocytochemistry, sections were incubated with primary antibodies and then with appropriate secondary antibodies conjugated to either fluorescein or rhodamine (Jackson ImmunoResearch, West Grove, PA). A monoclonal TH antibody, used at a dilution of 1:500, was purchased from Boehringer Mannheim. CaMKIV and b-gal antibodies were used at dilutions of 1:200 and 1:750, respectively, and covered with a double-stranded plasmid as the template based on published procedures (Inouye and Inouye, 1987) as described previously (Min et al., 1996).

Construction of TH8.9kb AP-1 mutant/lacZ fusion DNA. The original TH8.9kb/lacZ DNA construct (Mittnacht, 1994) was used to generate a TH8.9kb AP-1 mutant/lacZ fusion DNA construct by replacing the 0.2 kb XbaI-BglII DNA fragment containing the TH AP-1 site (5'-TGATTCA-3') located at ~200 bp upstream of the start codon with a 0.2 kb XbaI-BglII fragment containing two mutated nucleotides (underlined) (5'-TGTTA-3'). To generate the AP-1 double mutation, site-directed mutagenesis was performed using double-stranded plasmid as the template based on published procedures (Inouye and Inouye, 1987) as described previously (Tinti et al., 1997). DNA sequencing analysis confirmed the AP-1 double mutation. The four transgenic lines generated, reporter gene expression was similar in three lines (lines 7, 19, and 20), as described previously. The fourth exhibited ectopic glial staining and reporter gene expression was similar in three lines (lines 7, 19, and 20), as described previously.

For localization of a single antigen, tissue was blocked with 1% bovine serum albumin and 0.2% Triton X-100 in PBS and incubated overnight with primary antisera. The TH rabbit antibody (1:50,000 final concentration) was raised in our laboratory. Rabbit antibacterial b-galactosidase (b-gal) (1:5,000) was obtained from ICN Pharmaceuticals (Aurora, OH), monoclonal CaM kinase II (CaMKII) (0.2 mg/ml) was purchased from Boehringer Mannheim (Indianapolis, IN), and CaMKIV (CaMKIV) (1:2,000) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antigens were visualized by incubation with appropriate biotinylated secondary antisera and the Vector Elite kit (Vector Laboratories, Burlingame, CA) and 3,3'-diaminobenzidine (0.05%) and hydrogen peroxide (0.003%) as chromogen. Sections were mounted on slides, dehydrated through graded alcohols, and coverslipped.

In situ hybridization. Mice were perfused as described above except that all solutions used after fixation were prepared in DEPC-treated water. Sections (40 m) were collected in either 2° (for radiolabeling) or 4° (for digoxigenin-labeling) SSC in vials on ice. For radiolabeling of mRNA, sections were hybridized with a 1.6 kb TH probe labeled by random priming with 35S-dATP (107 dpm/ml) as described previously (Cho et al., 1996). Hybridization buffer contained 2° SSC, 15% formamide, 1° Tris EDTA dextran sulfate (TED), 1.6 mg/ml salmon sperm DNA, and 4° Denhart’s solution. Hybridization occurred overnight at 48°C. Tissue was washed, mounted on slides, apposed to Kodak X-Omat film (Eastman Kodak, Rochester, NY), and then dipped in nuclear-type B2 emulsion for ~2 weeks at 4°C. Slides were developed, counterstained, dehydrated, and coverslipped. For preparation of digoxigenin-labeled probes, template DNA was linearized and transcribed with T7 RNA polymerase for the antisense orientation. The hybridization buffer contained 4° SSC, 50% formamide, 0° TED, 0.56 mg/ml of salmon sperm DNA, 4° Denhart’s solution, and 250 µg/ml transfer RNA. After hybridization overnight at 60°C, sections were washed at 65°C. Message was demonstrated by either an alkaline phosphatase- or fluorescein-labeled secondary antibody to digoxigenin (Boehringer Mannheim). The chromogen [0.33 mg/ml nitroblue tetrazolium (NBT) and 0.16 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP)] was used to detect alkaline phosphatase.

X-Gal staining. Tissue sections were incubated overnight with a solution containing 3.1 mm potassium ferricyanide, 3.1 mm potassium ferrocyanide, 0.15 m NaCl, 1 mm MgCl2, 0.01% sodium deoxycholate, 0.02% Nonidet P-40, and 0.2 mg/ml X-Gal in 10 mm PB, pH 7.4, as described previously (Min et al., 1996).

b-Gal assay. A b-gal assay kit (catalog no. GAL-A; Sigma, St. Louis, MO) was used according to the manufacturer’s directions. OBs and SNs were obtained by regional brain dissections. The SN was composed of the midbrain ventral to the cerebral aqueduct. Because the rostral limit was the mammillary bodies and the posterior limit was midpons, there was a considerable dilution of the ~15,000 DA cells in the SN compared with...
RESULTS

Distribution of TH protein and mRNA in adult mouse olfactory system

In agreement with previous findings (Baker et al., 1983; Baker and Farbman, 1993), TH protein, as demonstrated by immunostaining, occurred primarily in periglomerular and tufted cells distributed around the glomeruli of the main olfactory bulb (Fig. 1A). The intraglomerular processes of these cells also displayed high levels of TH immunoreactivity (Fig. 1B, inset). Infrequently, immunostained cells were found in the external plexiform and mitral cell layers. The glomerular region of the accessory olfactory bulb (AOB) also contained a few TH-immunoreactive cells. TH protein could not be demonstrated within either the RMS (Fig. 1A) or the SVZa. TH mRNA, assessed by in situ hybridization using 35S-radiolabeled TH probe (Fig. 1C) or digoxigenin-labeled TH probe (Fig. 2), exhibited high levels of expression associated with the periglomerular region surrounding the glomeruli of the main OB. Less dense accumulations of either silver grains or reaction product, indicative of low TH mRNA levels, were found in the mitral and superficial granule cell layers (Figs. 1D, 2B, arrows). The external plexiform layer contained a few cells with TH mRNA (Fig. 2B, arrowhead). TH mRNA was also detected in the AOB (Fig. 2A, arrowhead).

lacZ expression in transgenic mice

The pattern of expression of the 8.9 kb TH/lacZ construct in the transgenic mice was the same when demonstrated by either X-Gal histochemistry or β-gal immunocytochemistry (Fig. 3A,C). The distribution of stained periglomerular cells and their intraglomerular processes were similar to those observed with TH antibodies. In the mitral cell layer, numerous small, granule-like cells could be demonstrated with both reporter gene detection procedures (Fig. 3B,D). The leading processes of the cells traversed the external plexiform layer to terminate either at or near the glomeruli, giving the impression that the cells were migrating toward the glomerular layer (Fig. 3D, inset). Definitive determination of intraglomerular terminations was difficult because of the presence of the heavily stained periglomerular cell processes. Superficially located granule cells also were labeled with β-gal and X-Gal (Fig. 3B,D). These cells often showed leading processes directed toward the external plexiform layer, again suggesting that they were presumptive dopaminergic neurons migrating toward the glomerular layer (Figs. 3B,D, 4B). The small but consistent number of transgene-expressing cells observed in the external plexiform layer suggested rapid migration through this layer. TH mRNA (Figs. 1C, 2A) and transgene (Fig. 3C) showed parallel distributions in the periglomerular and mitral cell layers. In the AOB, β-gal- and X-Gal-labeled cells were numerous in the granule and mitral cell layers, infrequent in the glomerular layer (Fig. 3A,C), and never associated with cellular TH immunostaining.

Characterization of the β-gal cells in the mitral cell layer

To determine whether the β-gal-immunolabeled cells in the mitral cell layer were granule or mitral cells, sections were stained with a calcium–calmodulin-dependent protein kinase II (CaMKII) antiserum (Fig. 4). Mitral cells, containing heavy cytoplasmic CaMKII staining, were clearly larger (~30–40 μm in diameter) than the β-gal-labeled cells (~10 μm). Granule cells, which were also immunolabeled with CaMKII, displayed only a thin rim of cytoplasmic staining.
Previous studies established that DA cells in the OB (Liu, 2000) and SN (our unpublished observation) did not contain CaMKIV immunoreactivity. The current study found that other β-gal-containing regions of the OB, the mitral and superficial granule cell layers, were also distinguished by their lack of labeling with antisera to CaMKIV (Fig. 5A). Double-labeling studies confirmed that β-gal and CaMKIV were expressed by different granule cell populations (Fig. 5B,C), suggesting that the dopaminergic phenotype may be determined before cells reach the periglomerular region. CaMKIV staining was limited to a small population of cells in the RMS (data not shown).

**Colocalization of β-gal, TH immunoreactivity, and TH mRNA**

Two populations of periglomerular cells could be distinguished using rhodamine immunofluorescence for β-gal and fluorescein immunofluorescence for TH observed by confocal microscopy (Fig. 5D,E). As expected, most cells appeared yellow when images were merged, demonstrating that they contained both β-gal and TH (Fig. 5F). A few periglomerular cells contained only β-gal immunoreactivity (Fig. 5D–G). These cells were more numerous in the periglomerular region either adjacent to or within the external plexiform layer. All β-gal-immunopositive cells in the mitral and granule cell layers exhibited only red (rhodamine immunofluorescence) because they did not contain TH protein (Fig. 5G).

Double-label studies localized TH mRNA with a probe prepared with fluorescein-labeled anti-digoxigenin and β-gal protein with rhodamine-labeled secondary antibodies; these studies showed that the β-gal-containing cells in the mitral cell layer expressed low but significant levels of TH mRNA (Fig. 5H–J,H′–J′). β-Gal immunoreactivity was somewhat reduced by the high temperature required for the in situ hybridization procedure. In contrast, TH immunoreactivity was restricted to periglomerular cells and their processes within glomeruli. TH mRNA was not observed in either the SVZa or the RMS with the fluorescent technique (data not shown).

**TH and β-gal expression after olfactory deprivation**

As published previously (Min et al., 1994), β-gal expression, especially in the intraglomerular processes of the periglomerular cells, declined in parallel with TH immunoreactivity (data not shown) in the periglomerular region of the OB ipsilateral to unilateral naris closure (Fig. 6A,B). However, the number of β-gal-immunoreactive cells expressed per area in the medial periglomerular region was higher in the OB ipsilateral compared with contralateral to naris closure (mean number of cells per mm$^2$ ± SE was 1372 ± 58.7 versus 923.2 ± 16.0; p < 0.002, respectively). Nasal closure produced a significant decline in the area of the OB [mean area in mm$^2$ ± SE was 2.43 ± 0.03 (ipsilateral) versus 3.27 ± 0.11 (contralateral); p < 0.001]. Thus, there was no difference in the number of periglomerular cells when adjusted for the change in OB area [total mean number of cells ± SE was 3381 ± 141 (ipsilateral) versus 3022 ± 95 (contralateral); p > 0.05]. The opposite pattern was observed in the mitral and granule cell layers, where β-gal immunoreactivity did not appear to be altered by naris closure. Expressed per unit area, the number of β-gal-immunoreactive cells in the mitral cell layer was similar [mean number of cells per mm$^2$ ± SE was 6134 ± 1000 (ipsilateral) versus 6840.2 ± 864 (contralateral); p > 0.05]. When corrected for OB shrinkage, the number of granule cells in the mitral cell layer was reduced by ~30% ipsilateral to closure [total mean number of cells was 14,905 ± 2741 (ipsilateral) versus 22,366 ± 2048 (contralateral); p < 0.01].

**β-Gal expression in transgenic mice with a mutated AP-1 site**

Three lines of transgenic mice were derived that expressed a transgene in which two bases were mutated in the context of the 8.9kb TH promoter. In the OB, transgene expression in mice expressing the mutant AP-1 construct was absent in the mitral cell layer and significantly reduced in the periglomerular region (Fig. 7A–D). In agreement with observations in transgenic mice expressing the normal TH promoter construct, no staining was observed in either the SVZa or RMS (data not shown). β-Gal
activity, measured in OB homogenates, was 4- to 15-fold lower in the mutant mice compared with the mice expressing the normal promoter construct (Fig. 7E). β-Gal immunoreactivity in the SN and locus ceruleus was similar in the control and mutant transgenic mice (data not shown). Assessment of β-gal levels in tissue homogenates of the SN (Fig. 7F) showed that transgene expression was not significantly different between the control strain and two of the strains with the mutant construct (AP-1m#19 and AP-1m#7) but that this expression was threefold higher in the third mutant strain (AP-1m#20). Because this latter strain displayed the lowest β-gal activity in the OB, the results strongly support the concept that TH regulation through the AP-1 site shows brain region-specific regulation.

**DISCUSSION**

Evidence is presented to support the hypothesis that DA phenotypic expression, previously recognized only in the glomerular layer of the OB (McLean and Shipley, 1988; Baker and Farbman, 1993), can be demonstrated in the migratory pathway before the interneurons attain their final periglomerular position. Adult transgenic mice that express an 8.9kb TH promoter–lacZ reporter construct displayed staining for β-gal, the lacZ gene product, in cells of the mitral and superficial granule cell layers using both X-Gal histochemical and β-gal immunohistochemical techniques. CaMKII immunostaining demonstrated that the β-gal-labeled cells in the mitral cell layer were granule cells. β-Gal-stained cells were not found in either the SVZa or the RMS. The paucity of β-gal labeling in the external plexiform layer, despite the fact that neurons clearly must traverse this layer, suggested that migrating cells move rapidly from the mitral cell to the glomerular layer. Although previous studies suggested that TH mRNA and protein occurred only in the periglomerular layer in association with odor-induced activity, the presence of mRNA in the mitral cell layer could be discerned, in fact, by closer inspection of published micrographs (Stone et al., 1991; Min et al., 1996). The long half-life of the bacterial gene product β-gal in the mammalian cells may contribute to the greater number of β-gal-containing cells than both TH mRNA- and protein-containing cells.

Double-labeling studies demonstrated that almost all periglomerular cells that expressed TH protein also had β-gal immunoreactivity. Some cells, primarily adjacent to the external plexiform layer, contained only β-gal immunoreactivity. The lack of TH protein may reflect either newly migrating cells not yet receiving innervation or cells that have been deafferented as a consequence of normal receptor cell turnover (Graziadei and Monti Graziadei, 1980). TH mRNA but not TH protein could be demonstrated in the β-gal-immunoreactive cells in the mitral cell layer, confirming the occurrence of TH transcription but not translation. A recent study used an antigen retrieval system to suggest that low levels of TH protein may be found in the mitral cell layer in association with a TH-reporter gene construct (Schimmel et al., 1999). Perikaryal labeling is difficult to discern in the low magnification micrographs and could represent either fiber terminations from centrifugal noradrenergic innervation (McLean and Shipley, 1991) or the unmasking of an antigen other than TH. TH-immunostained cells could be demonstrated in the mitral cell layer of neonates (Baker and Farbman, 1993) but not in the mitral cell layer of adults (Baker et al., 1983, 1993; McLean and Shipley, 1988; Baker, 1990; Stone et al., 1990, 1991). Even assuming that low-level, leaky TH protein expression does occur, the data show that DA phenotypic differentiation is incomplete during migration.

The mechanisms that produce the dissociation between TH mRNA and protein expression are as yet unknown. Previous studies have clearly established that receptor afferent stimulation is necessary for full phenotypic expression of the DA phenotype. The current data suggest that a very low level of stimulation is sufficient to induce transcription, but not translation of TH (see below). Evidence also exists for regulation of TH mRNA levels by factors binding to the 3′ untranslated region, supporting the notion that altered mRNA stability may contribute to these findings, especially in view of the low mRNA levels observed in superficial granule cells (Kroll et al., 1999; Makeyev et al., 1999).

The finding that at least partial DA differentiation occurred before interneurons reached the glomerular layer raised the issue of where periglomerular and granule cells initially attain their separate phenotypes, because all interneurons migrate in the RMS. Staining for CaMKIV, shown previously to phosphorylate the transcription factor cAMP response element-binding protein that binds to the cAMP response element sites in the promoters of many genes, including TH, was used to begin to address this issue (Enslen et al., 1994; Matthews et al., 1994). CaMKIV immunostaining revealed two distinct populations of interneurons during their migration through the OB, a population with
CaMKIV, β-gal, and TH protein and mRNA expression in the olfactory bulb. A, CaMKIV immunostaining is restricted to deep granule (gr) cells. Low- (B) and high- (C) magnification confocal images of sections double-labeled for β-gal (red) and CaMKIV (green) illustrate the complete separation of the two antigens. Sections immunostained with β-gal (D, red) and TH (E, green) show the large degree of overlap between transgene and TH protein expression in the glomerular (g) region of the olfactory bulb. In the merged image (F), cells containing both antigens are yellow. G shows that, in contrast to the colocalization of TH and β-gal protein in the glomerular layer, only β-gal immunostaining is observed in the mitral (m) cell layer and few cells exhibit staining in the external plexiform layer (epl). The colocalization of TH mRNA and β-gal protein is shown in H–J. Double-labeled cells appear yellow (arrows in H–J), H′–J′ are higher-magnification images of the mitral cell layer showing that TH mRNA (arrows in J′) but not TH protein is present in granule cells. Scale bars: A, B, 130 μm; C, H–J, 70 μm; D–F, 40 μm; G, 90 μm; H′–J′, 20 μm.

β-gal immunoreactivity and another that contained only CaMKIV. Few if any CaMKIV-immunoreactive cells were observed in the RMS, suggesting that phenotypic determination may not occur until interneurons reach the granule cell layer, but before the putative DA neurons reach the periglomerular region. These findings do not rule out the possibility that differentiation may be initiated either as stem cells divide in the SVZa (Doetsch et al., 1999a,b) or at the time of terminal division during migra-
tion through the RMS (Lois and Alvarez-Buylla, 1993; Luskin, 1993; Menezes et al., 1995; Brock et al., 1998; Kirschenbaum et al., 1999).

Also addressed was whether odor deprivation, shown previously to reduce TH mRNA and protein levels (Baker, 1990; Stone et al., 1990, 1991; Baker et al., 1993; Cho et al., 1996), downregulated β-gal expression in both the glomerular and mitral cell layers. In agreement with previous findings (Baker et al., 1984, 1988; Stone et al., 1990, 1991), the number of β-gal-containing periglomerular cells in the OB ipsilateral compared with contralateral to naris closure was not altered when adjusted for the decrease in OB area. In contrast, a reduction was found in the number of β-gal-expressing cells in the mitral cell layer. Differential regulation in these two OB populations may occur because the level of stimulation of interneurons in the mitral compared with the periglomerular cell layer is normally lower and is reduced even further after naris closure. The resulting level of trans-synaptic activation is thus below the threshold for transcriptional induction of β-gal, and presumably TH, in the interneurons of the mitral cell layer. The findings suggest that low-level stimulation may continue in the periglomerular region. Supporting this hypothesis is the significant loss of β-gal-stained processes in the glomeruli and the maintenance of perikaryal staining. A concurrent mechanism may be increased migration of neurons from the mitral to the periglomerular cell layer as a consequence of apoptotic cell loss in the glomerular layer (Najbauer and Leon, 1995; Fiske and Brunjes, 2001). Because previous studies suggested unchanged interneuron migration in the RMS after either naris closure or even bulbectomy (Frazier-Cierpial and Brunjes, 1999).


