

Spatiotemporally separable *Shh* domains in the midbrain define distinct dopaminergic progenitor pools

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Edited by Matthew P. Scott, Stanford University School of Medicine, Stanford, CA, and approved September 14, 2009 (received for review April 20, 2009)

Midbrain dopamine neurons (mDA) are important regulators of diverse physiological functions, including movement, attention, and reward behaviors. Accordingly, aberrant function of dopamine neurons underlies a wide spectrum of disorders, such as Parkinson's disease (PD), dystonia, and schizophrenia. The distinct functions of the dopamine system are carried out by neuroanatomically discrete subgroups of dopamine neurons, which differ in gene expression, axonal projections, and susceptibility in PD. The developmental underpinnings of this heterogeneity are undefined. We have recently shown that in the embryonic CNS, mDA originate from the midbrain floor plate, a ventral midline structure that is operationally defined by the expression of the molecule *Shh*. Here, we develop these findings to reveal that in the embryonic midbrain, the spatiotemporally dynamic *Shh* domain defines multiple progenitor pools. We deduce 3 distinct progenitor pools, medial, intermediate, and lateral, which contribute to different mDA clusters. The earliest progenitors to express *Shh*, here referred to as the medial pool, contributes neurons to the rostral linear nucleus and mDA of the ventral tegmental area/interfascicular regions, but remarkably, little to the substantia nigra pars compacta. The intermediate *Shh*+ progenitors give rise to neurons of all dopaminergic nuclei, including the SNpc. The last and lateral pool of *Shh*+ progenitors generates a cohort that populates the red nucleus, Edinger Westphal nucleus, and supraoculomotor nucleus and cap. Subsequently, these lateral *Shh*+ progenitors produce mDA. This refined ontogenetic definition will expand understanding of dopamine neuron biology and selective susceptibility, and will impact stem cell-derived therapies and models for PD.

dopamine | Sonic hedgehog | lineage | substantia nigra

The midbrain dopaminergic neurons (mDA) are involved in diverse physiological pathways, including motor control, cognition, and reward behaviors. These neurons are clustered in 3 anatomically defined areas: the substantia nigra pars compacta (SNpc), the ventral tegmental area (VTA), and the retrorubral fields (RRF) (1, 2). The SNpc cluster neurons project predominantly to the dorsolateral striatum and are involved in motor function. The VTA neurons project to the prefrontal cortex, amygdala, and nucleus accumbens, circuits involved in emotional and cognitive functions (1, 2). In addition to the anatomical and functional differences, transcriptome as well as marker analyses of neurons in mDA clusters have also revealed some molecular differences (2–5). Finally, neurons in the SNpc are selectively lost in Parkinson's disease (PD) patients (6, 7), as well as in various genetic mouse models (8–11), further highlighting the physiological diversity of mDA. However, little is known about whether mDA diversity is encoded at the progenitor cell level.

Recent lineage studies have revealed a surprising embryonic origin of mDA at the ventral midline of the midbrain (10, 12–15). Using a *Shh::Cre* driver, we have definitively shown that most mDA are derived from the floor plate (FP), operationally defined by the expression of *Shh* (10, 15). However, dynamic *Shh*

expression spans a broad domain, and thus it remains to be determined whether this domain is parceled into microdomains, each giving rise to unique cell types. Here, we ask (i) whether different mDA clusters are generated from distinct progenitors and (ii) whether other types of neuron clusters are derived from distinct *Shh*-expressing progenitors. We extend our previous analyses and provide a comprehensive study describing *Shh* descendents in the midbrain. Further, using dynamic spatial changes in the *Shh* expression domain, in conjunction with a genetic inducible fate mapping (GIFM) technique (16), we generate an embryonic progenitor map of the ventral midbrain. By spatially and temporally mapping diverse progenitor domains in the embryonic midbrain, we uncover distinct progenitor pools with differential proclivity to generate mDA clusters.

Results

***Shh* Expressing Progenitors Give Rise to Multiple Neuron Types in the Midbrain.** Our initial fate-mapping experiments using a *Shh::Cre* driver revealed that, in addition to mDA, other neuron types also originated from *Shh*+ progenitors (15). To define the repertoire of neurons derived from the *Shh* domain, we examined postnatal *Shh::Cre;R26R* midbrain sections (Fig. 1). Labeled cells were observed in the mDA clusters RRF, SNpc, and VTA/interfascicular nucleus (IF), in the rostral linear nucleus (RLi), medial accessory nucleus (MA3), red nucleus (RN), Edinger Westphal nucleus (EW), and supraoculomotor nucleus and cap (Su3). Some nuclei, such as the nucleus of Darkschewitsch (Dk) and the interstitial nucleus of Cajal (InC), were located just outside the large swath of Xgal-stained cells. Remarkably, some nuclei located even within or more ventral to the general Xgal-stained territory were conspicuously unlabeled. For instance, only sparse Xgal+ cells were visible in the substantia nigra pars reticulata (SNpr) and oculomotor nucleus (3N). No Xgal+ cells are observed in the interpeduncular nucleus (IP; Fig. 1 A–C). Therefore, neurons in these nuclei must originate outside the *Shh*+ domain.

The *Shh* Domain Encompasses Several Spatiotemporally Distinct Progenitor Domains. To determine how different *Shh*+ progenitors were allocated to assume disparate mDA and other neuronal fates, we took advantage of the fact that the *Shh* expression domain in the midbrain is dynamic. *Shh* is expressed initially at the ventral midline at the 6- to 8-somite stage in the mesodien-

Author contributions: M.J. and R.A. designed research; M.J., A.A., A.R., L.C., and B.Y. performed research; M.J. and R.A. analyzed data; and M.J., R.K., R.M., and R.A. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0904285106/DCSupplemental.

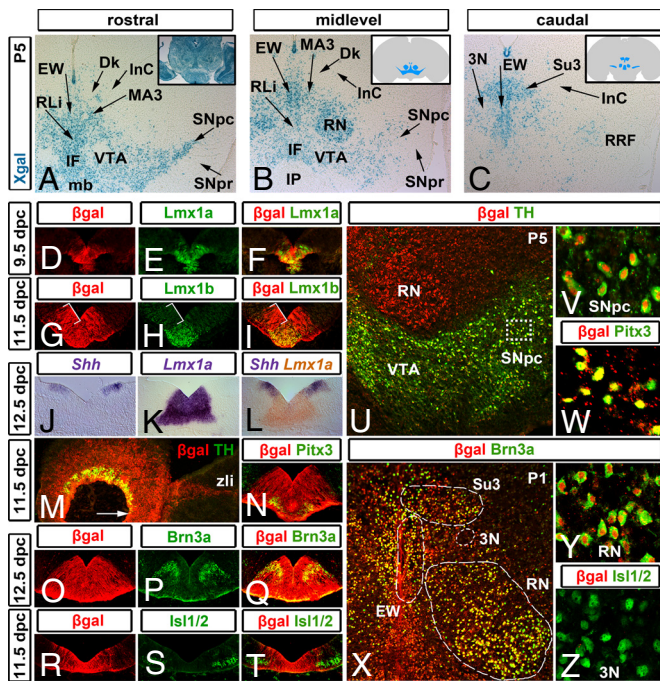


Fig. 1. *Shh*⁺ progenitors give rise to several distinct midbrain neuron populations. (A–C) In *Shh::Cre;R26R* P5 coronal sections at rostral (A), mid (B), and caudal (C) midbrain levels, Xgal-labeled cells were detected in the retrorubral fields (RRF), the substantia nigra pars compacta (SNpc), the ventral tegmental area (VTA)/interfascicular nucleus (IF) regions, the rostral linear nucleus (RLi), the Edinger Westphal nucleus (EW), the supraoculomotor nucleus and cap (Su3), the red nucleus (RN), and the medial accessory oculomotor nucleus (MA3). Conversely, the oculomotor nucleus (3N), the substantia nigra pars reticulata (SNpr), the nucleus of Darkschewitsch (Dk), the interstitial nucleus of Cajal (InC), and the interpeduncular nucleus (IP) were predominantly devoid of Xgal-labeled cells. In control *β-actin::Cre;R26R* brains, all midbrain nuclei were labeled (A Inset). (D–I) Embryonic midbrain coronal sections labeled with antibodies that recognize *β-gal* (*Shh* fate-mapped cells; red) and *Lmx1a* (E and F) or *Lmx1b* (H and I) (green). Early *Lmx1a*⁺ progenitors largely colocalize with *β-gal*⁺ cells (D–F). During dopaminergic neurogenesis, all *Lmx1a*/*Lmx1b*⁺ progenitors are situated within the *β-gal*⁺ territory. Immediately lateral to the *Lmx1a*/*Lmx1b*⁺ progenitor pool, an *Lmx1a*/*Lmx1b*[−], *β-gal*⁺ domain (brackets) predominantly produces other neuron types at these stages (G–I). (J–L) Complementary pattern of *Shh* and *Lmx1a* mRNA at 12.5 dpc. Double in situ hybridization for *Shh* (purple)/*Lmx1a* (orange) (L) depicts that the lateral *Shh* domain is largely *Lmx1a*[−], with some sections showing 2- to 3-cell diameter overlap. (M) In 11.5-dpc sagittal sections, the first TH⁺ dopamine neurons are observed rostrally (arrow) roughly until the zona limitans intrathalamica (zli). In coronal 12.5-dpc sections, the *β-gal* domain is wider than but inclusive of the *Pitx3*⁺ neurons (M), whereas *Brn3a*⁺ neurons are primarily embedded within the lateral aspects of the *β-gal* domain (O–Q). *Isl1/2*⁺ oculomotor neurons (3N) reside largely outside of the *β-gal* domain (R–T) at 11.5 dpc. (U–Z) Postnatal coronal midbrain sections were labeled with *β-gal* (U–Z; red) and TH (U and V), *Pitx3* (W), *Brn3a* (X and Y), or *Isl1/2* (Z) (green). Note that most TH⁺ and *Pitx3*⁺ cells of the SNpc and VTA are *β-gal*⁺ (SNpc, 97.76% SEM ± 0.36; VTA, 98.34% ± 0.46; IF, 97.80% ± 0.65; RRF, 96.12% ± 1.16; RLi, 97.79% ± 0.49 determined as percentage of the *β-gal*⁺/*Pitx3*⁺ cells). Similarly, the majority of the *Brn3a*⁺ cohort that populates the RN, EW, and Su3 is also *β-gal*⁺ (RN, 95.81% ± 0.38; EW, 97.23% ± 0.53; Su3, 90.95% ± 2.35). Shown in (X) is a relatively rostral section, in which only the beginning of the Su3 is present. Only a few *Isl1/2*⁺ cells of 3N were *β-gal*⁺ (Z; 10.75% ± 1.72). (V) High-power image of the area indicated by the white box in (U). (Y and Z) High-power images from the regions outlined in (X). Anatomical definitions were in accordance with the mouse brain atlas of Paxinos (see Fig. S8). mb, mammillary body.

cephalic region. Shortly thereafter, expression appears to spread rostrally to the anterior brain, and caudally into the hindbrain and spinal cord regions (17). In *Shh::Cre;R26R* embryos 9.0 days postcoitus (dpc), Xgal labeling is observed at the midline of the

midbrain (supporting information (SI) Fig. S1). By 9.5 dpc, and through 10.5 dpc, *Shh* expression and consequent Xgal labeling expands laterally; this lateral expansion is first observed in rostral midbrain sections. Between 10.5 and 11.5 dpc, *Shh* continues expanding laterally but is downregulated at the midline in the *Lmx1a*⁺ progenitors (15, 17, 18). Other FP markers, such as *Netrin-1*, *F-spondin*, and *Foxa2*, are also expressed in a similarly broad domain (Fig. S1). Thus, the cumulative *Shh* domain encompasses a swath of cells that expresses several traditional FP markers, and accounts for 25%–30% of the dorsoventral axis of the neural tube.

We reasoned that the cumulative *Shh* domain encompassed progenitors for multiple distinct neuron types. The transcription factors *Lmx1a* and *Lmx1b* are coexpressed at the ventral midline of the midbrain and are critical for the normal mDA phenotype (19, 20). In coronal sections of *Shh::Cre;R26R* 9.5-dpc embryos, *β-gal*⁺ cells are colocalized with *Lmx1a* and *Lmx1b* (Fig. 1 D–F) (15). As the *Shh* domain expands laterally, *β-gal* immunoreactivity is observed in more lateral domains. Thus, by 11.5 dpc, the *β-gal* domain is wider than, but inclusive of, the *Lmx1a*/*Lmx1b* domain (Fig. 1 G–I). At 11.5 and 12.5 dpc, all TH⁺ as well as *Pitx3*⁺ neurons appear to emanate directly from *Lmx1a*/*Lmx1b*⁺ ventricular zone; none are observed emanating from lateral *β-gal*⁺, *Lmx1a*[−] ventricular zone (along the rostrocaudal axis, TH⁺ cells extend from the isthmus to the zona limitans). Hence, until 12.5 dpc, the lateral *β-gal*⁺, *Lmx1a*/*Lmx1b*[−] domain corresponds largely to the lateral *Shh* expression domain (Fig. 1 J–N) (15), whose descendants are predominantly not TH⁺ neurons. Because the RN was prominent in our fate map (Fig. 1B), we tested whether these neurons may originate in the lateral aspects of the *Shh* domain, by double labeling with *Brn3a*, a marker for these neurons (21–23). Indeed, a *β-gal*⁺/*Brn3a*⁺ cohort was observed emanating from lateral *β-gal*⁺ progenitors (Fig. 1 O–Q). This cohort of *β-gal*⁺/*Brn3a*⁺ neurons populates not only the RN, but also EW and Su3 (Fig. 1 X and Y; elaborated further in Fig. 3).

The oculomotor nucleus and the GABAergic nuclei were largely unlabeled in our fate maps (Fig. 1 A–C). Indeed, *Isl1/2*⁺ 3N neurons, first emerging at 9.5 dpc, are observed immediately adjacent to the *β-gal*⁺ cohort, and few *Isl1/2*⁺ cells are colabeled with *β-gal* in 18.5-dpc sections (Fig. 1 R–T and Z). Additionally, a *GATA3*⁺/*Lim1/2*⁺ GABAergic cohort, first detected at 10.5 dpc, appears to at least in part originate from *Nkx2.2*⁺ progenitors. These progenitors and this postmitotic GABAergic cohort are observed outside the *β-gal*⁺ domain in *Shh::Cre;R26R* embryos (Fig. S2). Consequently, GABAergic nuclei are unlabeled in postnatal fate maps (Fig. 1 A–C).

***Shh* Domain Encompasses Distinct mDA Progenitor Pools with Different Prolivities.** Because the cumulative *Shh* domain is broad, we next sought to refine our fate maps by labeling distinct subsets of *Shh*⁺ progenitors. We therefore took advantage of the dynamic *Shh* expression domain; using an inducible *Shh::CreER^{T2}* strain (24), we were able to label spatiotemporally distinct subsets of *Shh*⁺ progenitors by administering tamoxifen (TAM) at different gestational times. Following TAM administration, for a period of up to 36 h (16), the cytoplasm-sequestered *CreER^{T2}* protein can translocate to the nucleus, and effectively recombine *loxP* sites in the *R26R* locus.

TAM administration at 7.5 dpc labeled the most medial progenitors at the ventral midline. In 7.5 (injection) → 10.5-dpc (harvest) *Shh::CreER^{T2};R26R* embryos, Xgal cells appeared to occupy only the most medial region of the *Lmx1a* domain (hereafter called the medial domain; Fig. 2A); adjacent *Lmx1a*⁺ progenitors seemed largely Xgal[−] (hereafter called the intermediate domain). This suggests that functional TAM activity in this context was extinguished before the expansion of the *Shh* domain observed at 9.5 dpc (Fig. S1). Further, these data also

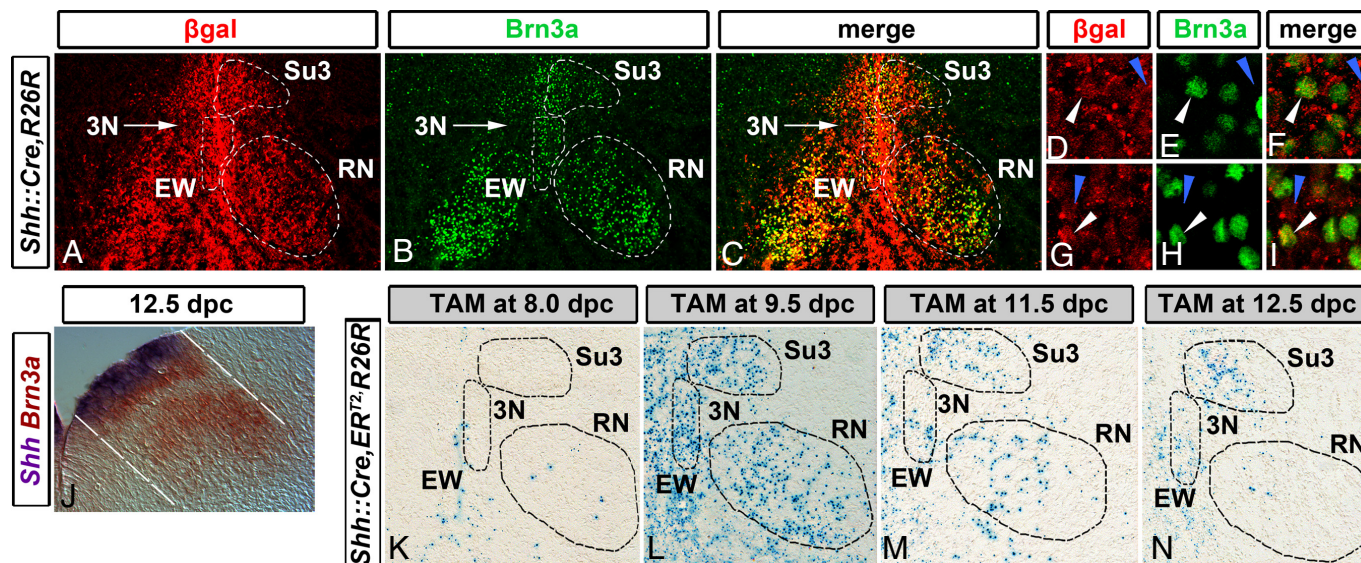


Fig. 3. Lateral *Shh*+ progenitors give rise to *Brn3a*+ neurons. (A–J) Labeling of P1 *Shh::Cre, R26R* coronal sections with antibodies that recognize β -gal (red) and *Brn3a* (green) shows many colocalized neurons in the EW, Su3, and the RN (outlined by dotted white lines in A–C). (D–F) and (G–I) show high-power confocal images from the Su3 and EW nuclei, respectively. Though most cells in all 3 nuclei are β -gal+/*Brn3a*+ (white arrowheads), some are β -gal+/*Brn3a*- (blue arrowheads), and a few are β -gal-/*Brn3a*+. (J) Double in situ hybridization for *Shh* (purple)/*Brn3a* (orange) showing that *Brn3a* cohort appears to emanate from the lateral *Shh* domain, in accordance with previous models (23, 25). (K–N) *Shh::Cre, ER^{T2}, R26R* embryos were TAM injected at 8.0 (K), 9.5 (L), 11.5 (M), and 12.5 dpc (N) and harvested at 18.5 dpc. In 8.0-dpc injections, few to no Xgal-labeled cells are detected in the EW, Su3, and RN (outlined with dotted lines). In contrast, in 9.5- and 11.5-dpc injections, robust Xgal labeling is observed. In 12.5-dpc injections, very few cells were detected in the RN, and only caudal EW and Su3 regions contained labeled cells. 3N is largely unlabeled in all TAM-induced embryos.

no labeled cells were observed in these clusters (Fig. 3K). In contrast, in 8.5-, 9.5-, and 11.5-dpc-injected embryos (wherein lateral *Shh*+ progenitors are also recombined), several labeled cells were observed in all 3 clusters (Fig. 3L and M; Table S1). In 12.5-dpc-injected embryos, only few cells were detected in the RN, in accordance with the end of the RN neurogenetic interval (25); more caudally, several labeled cells were detected in the EW and Su3 regions (Fig. 3N). These data suggest that the *Brn3a* cohort is largely derived from the lateral, but not medial or intermediate, *Shh* domains.

Discussion

A key motivation for detailing the developmental underpinnings of mDA diversity is the exciting potential for stem cell-derived models and therapeutics for PD (26). First, iPS-derived mDA from PD patient fibroblasts have been reported and will likely serve as valuable in vitro human cell models for this disease (27). Second, attempts to rescue rodent models of PD with mDA derived from mouse embryonic stem (ES) cells that have been programmed with developmental regulatory genes have been successful (28–30). To further improve both of these techniques, it is critical to refine ES cell manipulations to specifically produce SNpc (A9)-type mDA, the neurons that are prominently lost in PD patients (31, 32). Thus, defining the embryonic progenitor pools that contribute to distinct subsets of mDA in vivo is an important step toward our understanding and treatment of diseases of the dopamine system.

The floor plate (FP) is a key ventral organizer in the CNS, operationally defined by the molecule *Shh*. Previously, we have shown that the hindbrain and spinal cord FP are neurogenetically inert, but surprisingly, the midbrain FP is highly neurogenic and generates mDA. Here, we extend these studies to show that in the embryonic midbrain, the *Shh*-expressing domain is dynamic, and that at different times the *Shh* domain defines progenitor populations with distinct proclivities to generate different neuron types (Fig. 4 and Fig. S8). Using conventional fate mapping in conjunction with a GIFM approach, we develop a

model in which the medial *Shh*+ progenitors give rise to mDA located in the VTA/IF regions, as well as RLi neurons. Remarkably, these medial progenitors do not contribute efficiently to the SNpc. In contrast, when medial and intermediate progenitors are labeled, both VTA/IF and SNpc dopamine neurons are generated. Lateral *Shh*+ progenitors define the primordium of a *Brn3a*+ cohort that populates the RN, EW, and Su3. Subsequently, some lateral *Shh*+ progenitors appear to produce mDA. Together, these data reveal that the midbrain FP is a dynamic organizer that makes extensive and temporally distinct cellular contributions to the tissue that it patterns, and in that way resembles another key *Shh*-expressing organizer—the ZPA of the limb (24).

The dynamic expansion of the midbrain *Shh* domain could conceivably occur by one of 2 mechanisms. First, it is possible that expansion of the *Shh* domain results from the growth of the initial *Shh*-expressing population. Second, it is possible that *Shh* expansion occurs by inductive mechanisms, such that the initial *Shh*-expressing population induces neighboring cells to express *Shh*. Here, by restricting labeling to medial progenitors, we reveal that these labeled progenitors do not expand concomitantly with the *Shh* domain. Thus, our data favors a model wherein the expansion of the *Shh*+ FP occurs primarily by inductive mechanisms rather than growth of the initial population. Although further studies will be required to define these inductive mechanisms in mouse, studies in zebrafish spinal cord have suggested that *Shh* itself, first expressed in the medial FP, may play an inductive role in the expansion of the FP (33).

Our data support the presence of 3 distinct dopaminergic progenitor pools (Fig. 4 and Fig. S8). A medial pool, at the immediate midline, gives rise to rostral linear neurons and some mDA neurons mainly in the VTA/IF regions. Strikingly, only few SNpc mDA are derived from this domain. When medial and intermediate *Shh*+ progenitors are labeled, SNpc neurons are observed. We deduce that SNpc neurons are largely derived from intermediate progenitors (Fig. 4 and Fig. S8). Because medial and intermediate domains roughly comprise the *Lmx1a/b*

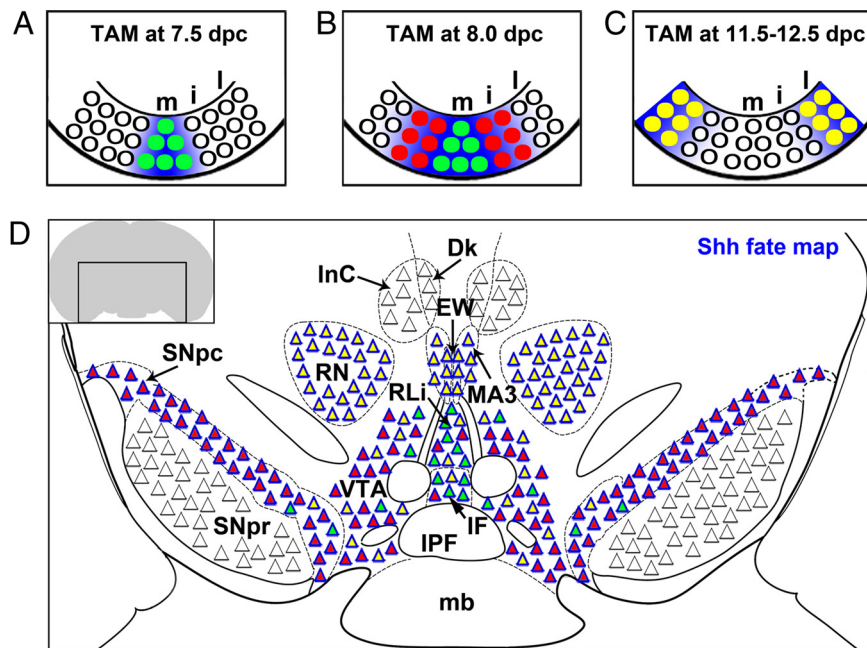


Fig. 4. A model for the embryonic origin of midbrain nuclei derived from spatiotemporally separable *Shh*⁺ progenitors. (A–C) Schematic representation of *Shh*⁺ domains during midbrain development. Based upon cumulative and inducible genetic fate-mapping results, we propose that midbrain *Shh*⁺ progenitors (green, red, and yellow circles) occupy 3 spatiotemporally separable domains: medial (green; *Lmx1a*⁺), intermediate (red; *Lmx1a*⁺), and lateral (yellow; largely *Lmx1a*[–]). Blue background approximates Xgal-labeled domains after indicated TAM injections. Note that the time of labeling of progenitors does not necessarily correlate to the birthdates of descendent neurons derived from those progenitors. (D) Schematic of the adult organization of major rostral midbrain nuclei with respect to their embryonic origin. The color within each triangle corresponds to the progenitor domain from which these neurons are derived. The medial progenitors (green circles in A and B) predominantly contribute to the RLi (mostly TH[–]) and sparsely to the VTA/IPF regions, but remarkably less to the SNpc. More caudally, these progenitors contribute few neurons to the VTA (see Fig. S8). The intermediate progenitors (red circles in B) contribute to the RLi and mDA in the VTA/IPF regions and SNpc. The lateral *Shh*⁺ progenitors (yellow circles in C) delineate the primordium of a *Brn3a*⁺ cohort that populates the EW, Su3 (see also Fig. S8), and the RN (this nucleus is largely unlabeled in 12.5-dpc TAM injections, but is derived from the lateral *Shh* domain at 10.5 and 11.5 dpc). The lateral progenitors also generate some mDA. Although in close proximity to *Shh* descendants (blue triangle contour) in postnatal sections, several populations are largely unlabeled (black triangle contour) in our experiments. Thus, these cells must be derived outside of *Shh*⁺ progenitors. These cells belong to the 3N (*Isl1/2*⁺), IP, and GABAergic neurons of the SNpr, PRN, Dk, and InC (see also Fig. S8). A schematic of a fate map at a more caudal level is shown in Fig. S8. IPF, interpeduncular fossa.

domain, our data suggest that *Lmx1a/b* progenitors are, at least, parceled into 2 subgroups having distinct proclivities. Consistent with this notion, molecular subdivisions have been observed within the *Lmx1a/b* territory. For example, high levels of *Corin* appear to define medial, but not intermediate, *Lmx1a*⁺ progenitors (34). In contrast, gene expression patterns of *Wnt1* and *RALDH1* appear to define mainly intermediate, but not medial, *Lmx1a*⁺ progenitors (13, 18). Further, *Wnt1*-derived fate maps do reveal neurons in the SNpc (13). A third, lateral, *Shh*⁺ progenitor domain is situated largely outside the *Lmx1a* domain. Although clonal analysis will be required for definitive verification, our data suggest that this lateral *Shh* domain generates a *Brn3a*⁺ cohort, and then appears to switch competence and give rise to a cohort of mDA that are restricted mainly to the VTA/IPF regions. In this regard, it is interesting to revisit studies involving conditional *Otx2* mutants, wherein large numbers of ectopic mDA appear to be generated laterally at the expense of the RN (35). *Otx2* may thus play a pivotal role in determining the competence of this lateral progenitor pool. Taken in the context of these studies, our data suggest that medial, intermediate, and lateral progenitors have unique intrinsic properties that result in the production of distinct cohorts of neurons.

Our fate maps reveal that nuclei with diverse functions, such as the *Brn3a*⁺ RN, EW, and Su3, are derived from the lateral *Shh* domain (Fig. 4 and Fig. S8). In contrast, the *Isl1/2*⁺ 3N cohort is relatively unlabeled in our fate maps. We posit a model in which a common progenitor pool first generates *Isl1/2*⁺ motorneurons and subsequently generates the *Brn3a* cohort, akin to the sequential generation of motor and serotonin neurons observed in the hind-

brain (36, 37). In our study, *Isl1/2*⁺ 3N neurons appear to arise from progenitors immediately adjacent to the *Shh* domain (at 9.5 dpc). As *Shh* expression spreads into these adjacent progenitors, *Isl1/2*⁺ neurons ceased to be produced. Instead, these progenitors, now included in the *Shh::Cre* recombinase domain, subsequently generate the *Brn3a*⁺ (β -gal⁺) cohort (10.5 dpc onward). This model is consistent with rodent birthdating studies showing that the neurogenetic interval of the 3N precedes that of the RN (25). Accordingly, the *Isl1/2*⁺ and *Brn3a*⁺ neurons are in register along the ventricular-pial axis of the 12.5-dpc embryo. As development proceeds, several fate-mapped *Brn3a*⁺ cells settle more ventrally than *Isl1/2*⁺ cells, forming the RN. Smaller cohorts of fate-mapped *Brn3a*⁺ cells populate the EW that lies medial to 3N, as well as the Su3, which remains dorsal to the 3N. Thus, the early emerging 3N is ultimately surrounded by the later emerging *Brn3a* cohort.

Using a genetic fate-mapping approach, we have defined distinct progenitor pools in the embryonic midbrain (Fig. 4 and Fig. S8). We reveal that different populations of mDA may be derived from distinct progenitors. Additionally, we propose the progenitor domains for several other important midbrain nuclei. Future refinements using other recombinase driver strains as well as intersectional genetic approaches (38, 39) would allow further dissection of the mDA primordium. These should also reveal progenitor distinctions along the rostrocaudal axis. Accurate embryonic midbrain maps will facilitate our understanding of the biology of the dopaminergic system, the interpretation of gain- and loss-of-function phenotypes of various mouse mutants, and the spectrum of human neurological conditions related to the neurotransmitter dopamine.

Materials and Methods

Mice. Mice bearing a *GFPcre* or *CreER^{T2}* fusion that was knocked in to the *Shh* locus (hereafter designated *Shh::Cre* or *Shh::CreER^{T2}*) (24) were crossed to *R26R* indicator mice. In inducible fate-mapping experiments, 4–9 mg/40 g TAM (dissolved in corn oil) was delivered by IP injection as previously described (40). Nine to 10 a.m. on the day of the vaginal plug was designated as 0.5 days postcoitum (dpc). For key time points, embryos from at least 2 litters were analyzed. Embryos were harvested and analyzed between 10.5 and 19.5 dpc. Mice were maintained and killed according to the protocols approved by the Northwestern Animal Care and Use Committee. Two embryos that received vehicle (oil only) showed no Xgal labeling in the brain.

Xgal Histochemistry, in Situ Hybridization, and Fluorescent Immunolabeling. Tissue preparation, detection of Xgal, *mRNA* in situ hybridization, and immunofluorescence were performed as described (15, 38). Antibodies

used include goat β -gal (Biogenesis), guinea pig Lmx1b, rabbit Lmx1a, rat BrdU (Serotec), rabbit and sheep tyrosine hydroxylase (Pel Freeze), rabbit Pitx3 (Zymed), rabbit GABA (Sigma), goat GATA3 (Santa Cruz), goat Foxa2 (Santa Cruz), rabbit Brn3a, mouse Isl1/2 [Developmental Studies Hybridoma Bank (DSHB)], mouse Lim1/2 (DSHB), mouse Nkx6.1 (DSHB), and mouse Nkx2.2 (DSHB). Epifluorescent or confocal images were processed in Adobe Photoshop CS2.

ACKNOWLEDGMENTS. We thank Dr. Cliff Tabin for *Shh::CreGFP* and *Shh::CreER^{T2}* mice and *Shh* cDNA; Dr. Michael German for anti-Lmx1a; Drs. Thomas Muller and Carmen Birchmeier for anti-Lmx1b; and Dr. Eric Turner for anti-Brn3a antibodies. We thank Dr. Doug Kim for useful comments and Dr. Abdelhak Belmadani and Bor-Shuen Wang for imaging and technical assistance. R.A. was supported by the Whitehall Foundation, Brain Research Foundation, and the American Parkinson's Disease Association. M.J. was supported by the Parkinson's Disease Foundation.

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