Supporting Information

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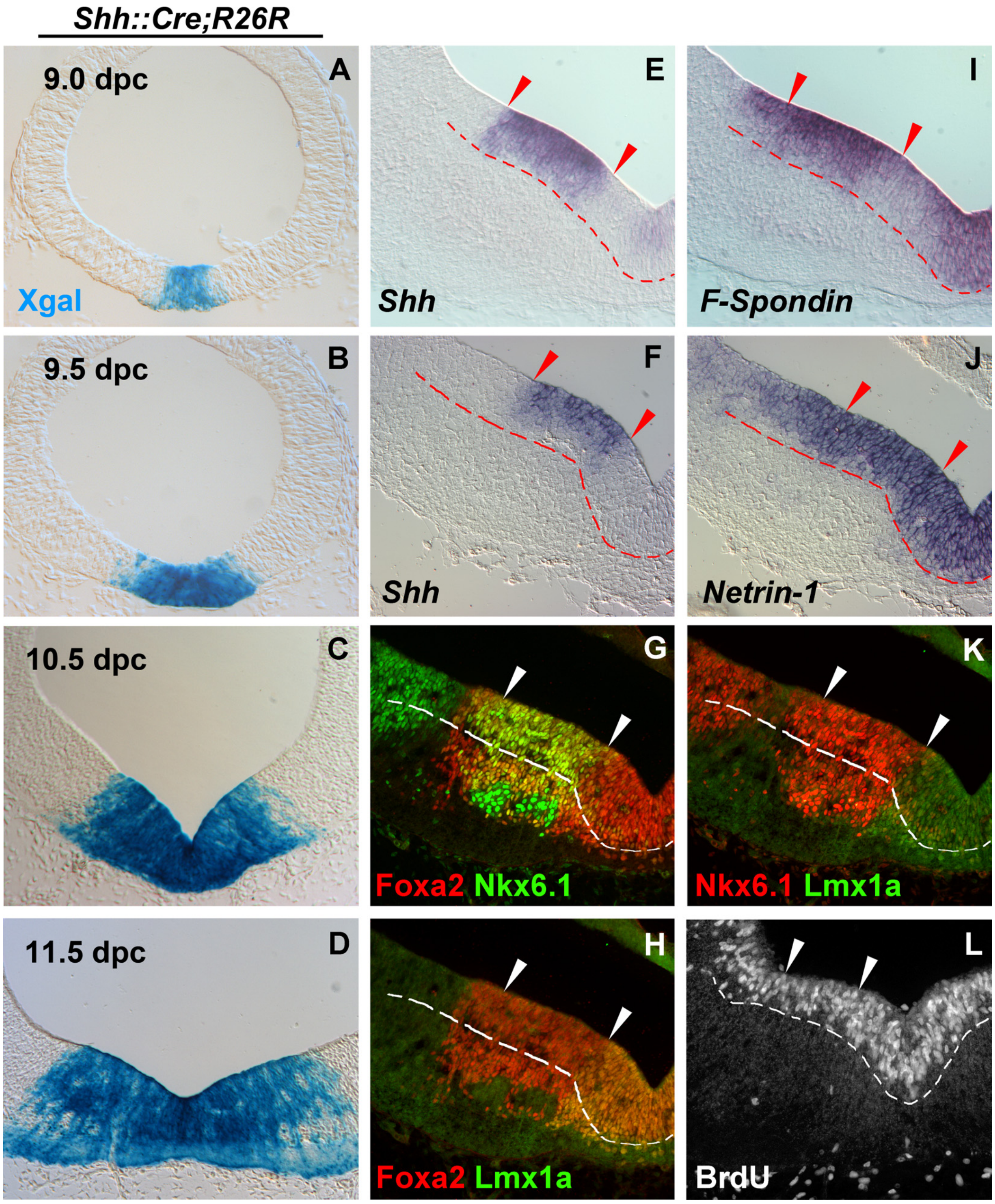

Fig. S1. Molecular characteristics of the *Shh* domain. (*A–D*) Lateral expansion of *Shh* during midbrain development. In *Shh*::*Cre,R26R* coronal midbrain sections, Xgal labeling is initially restricted at the immediate ventral midline at 9.0 dpc (*A*) and then flares out laterally between 9.5 and 11.5 dpc (*B–D*). At later stages, Xgal labeling is detected not only in the ventricular zone where *Shh* expression is confined (*E* and *F*), but also in the adjacent mantle layer, coinciding with an appearance of postmitotic neurons (see also Fig. 1). The cumulative *Shh* domain is characterized by expression of known floor-plate markers. (*E–H*) Coronal midbrain sections at 11.5 dpc showing *Shh* (*E* and *F*) predominantly in the lateral domain (in all sections the approximate position of the lateral *Shh* domain is demarcated by red or white arrowheads, and the dotted line demarcates the ventricular zone). *F-spondin* (*I*) is expressed throughout the ventral midline, although it is more robust in the region corresponding to the lateral *Shh* domain; expression also extends a few cells lateral to *Shh*. *Netrin-1* (*J*) is also expressed throughout the midline and extends lateral to *Shh*. Foxa2 (*G*) is expressed throughout the ventral midline, and appears to extend a few cells lateral to *Shh*. Nkx6.1 appears to largely coincide with the lateral *Shh* domain. The Nkx6.1 and Lmx1a domains (*K*) appear separate, although the domains overlap by 1–2 cells; further, faint Nkx6.1 is observed in many Lmx1a+ progenitors. The Foxa2 domain extends well beyond Lmx1a (*H*). (*L*) In 12.5-dpc embryo sections, many BrdU+ cells are observed throughout the ventral midbrain, suggesting that all these progenitor domains are still neurogenically active. Taken together, the *Shh* domain expands during development, expresses several floor-plate markers, and is mitotically active.

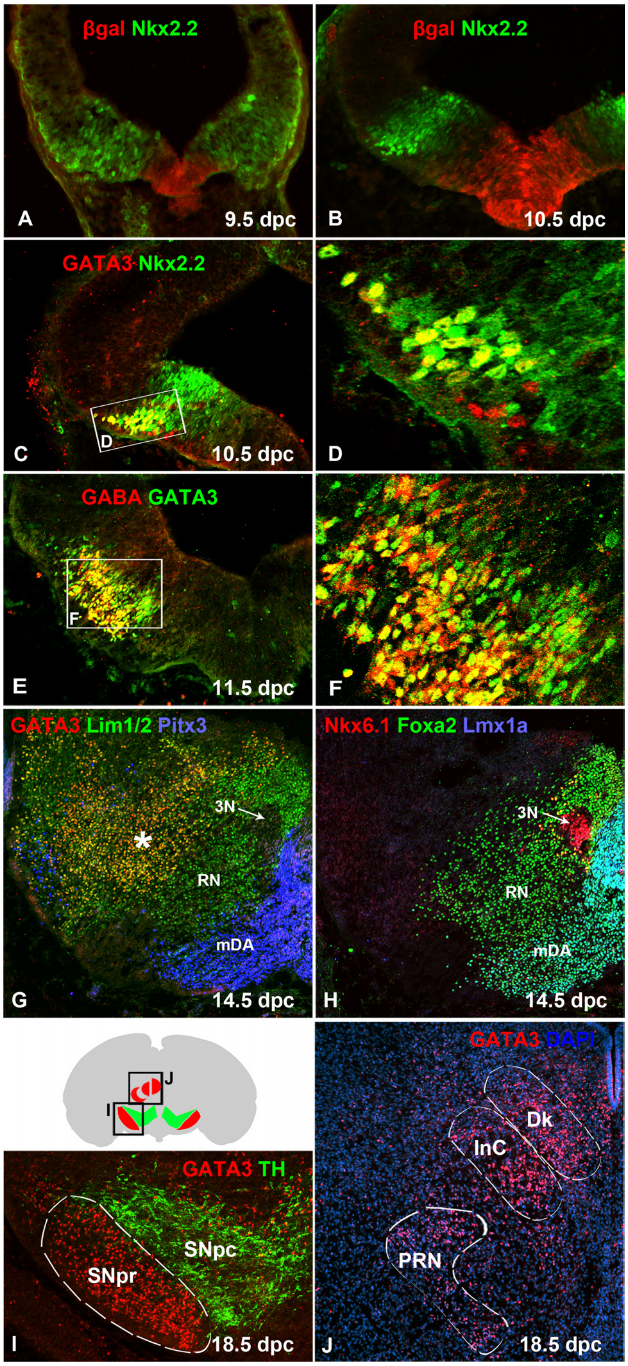

Fig. S2. Ventral midbrain GABAergic neurons are not *Shh* descendants and originate, at least in part, from the adjacent Nkx2.2+ domain. (*A* and *B*) Coronal sections of *Shh*::*Cre,R26R* embryos labeled with β-gal and Nkx2.2 shows that the *Shh*+ and Nkx2.2+ domains are mutually exclusive (*A* and *B*). (*C* and *D*) Double immunodetection of GATA3 (red) and Nkx2.2 (green) shows that many newly born Nkx2.2+ cells are GATA3+. (*D*) High-power confocal image of the white-boxed area indicated in (*C*). (*E* and *F*) Double labeling for GABA (red) and GATA3 (green). (*F*) High-power confocal image of the white-boxed area indicated in (*E*), showing several GATA3+/GABA+ cells. (*G*) Triple labeling on coronal sections with GATA3 (red)/Lim1/2 (green)/Pitx3 (blue) and (*H*) Nkx6.1 (red)/Foxa2 (green)/Lmx1a (blue) at 14.5 dpc. Note coexpression of GATA3 and Lim1/2 in the GABAergic cohort (asterisk) that is clearly separated from the Lim1/2+/Foxa2+ red nucleus (RN), Pitx3+/Foxa2+/Lmx1a+ midbrain dopamine neurons (mDA), and Nkx6.1+ oculomotor neurons (3N; arrows). (*I* and *J*) GATA3 appears to mark most ventral midbrain GABA+ neuron clusters, including the substantia nigra pars reticulata (SNpr) as well as the pararubral nucleus (PRN), the interstitial nucleus of Cajal (InC), and the nucleus of Darkschewitsch (Dk); in addition, scattered GATA3+ cells were also detected within the RN. DAPI is shown in (*J*), to demarcate the separation of the InC and Dk nuclei, the Dk being located within the region of the periaqueductal gray. These analyses suggest that progenitors of GABAergic neurons are positioned immediately lateral to the *Shh*+ domain; consequently, GABAergic nuclei are located largely outside of the *Shh* fate-mapped nuclei (see Fig. 1).

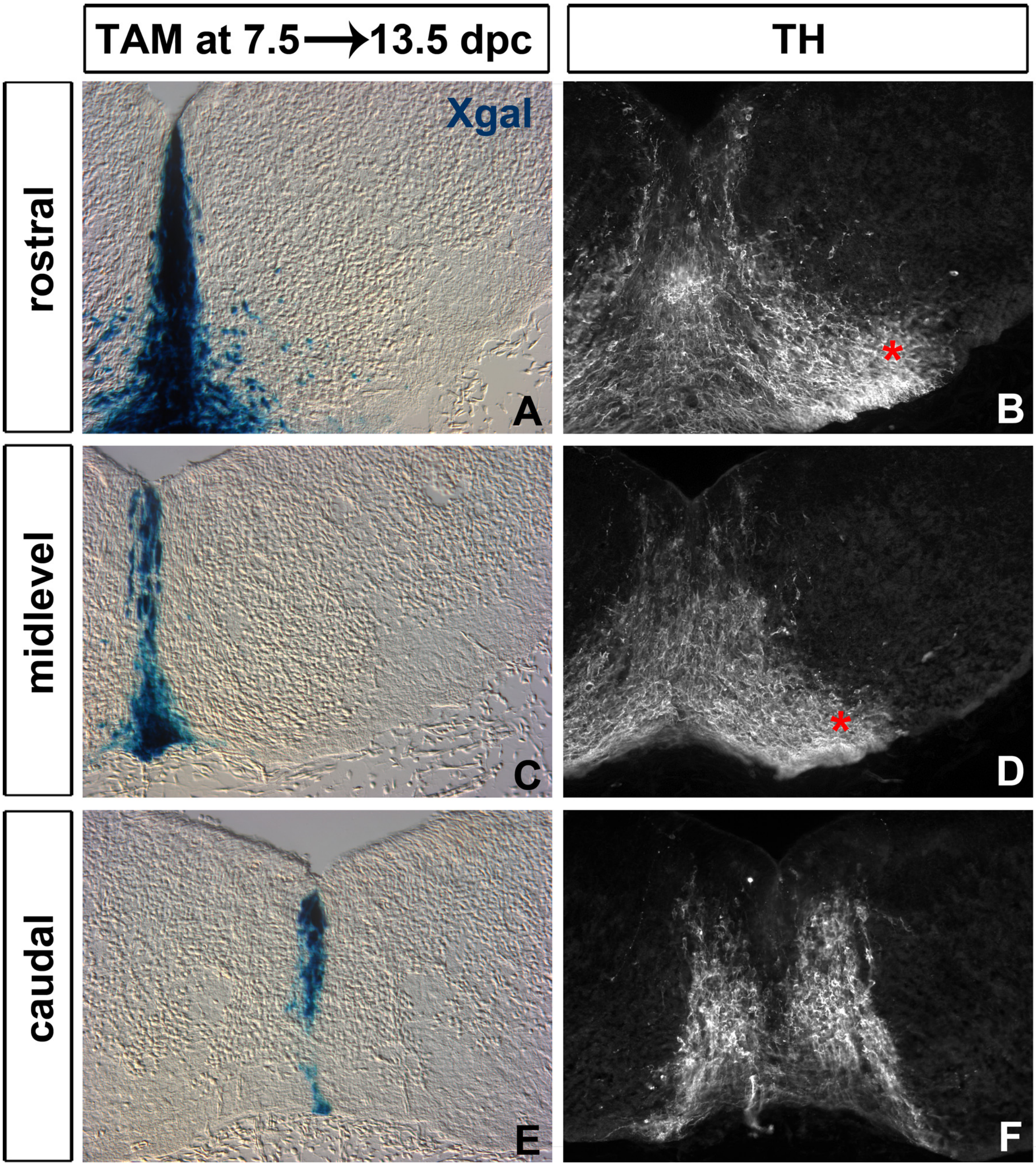

Fig. S3. Xgal and TH labeling on coronal midbrain adjacent sections along anterior posterior axes in 7.5 dpc tamoxifen-induced *Shh*::*CreER*^{T2},*R26R* embryos. (*A*–*F*) In 7.5 (injection) → 13.5 dpc (harvest), at rostral and midlevels, Xgal-labeled cells are observed emanating from medial progenitors into the medial aspects of the TH+ domain (*A*–*D*). Very few Xgal cells are observed in the lateral aspects of the TH+ domain (asterisks in *B* and *D*). At caudal levels, only few Xgal-labeled cells are detected in the mantle, and only within the medial aspects of TH+ domain (*E* and *F*). These findings support a model wherein rostrally, the medial progenitor domain contributes little to the SNpc. Furthermore, the caudal medial progenitor domain may be narrower, and perhaps less neurogenic than its rostral counterpart.

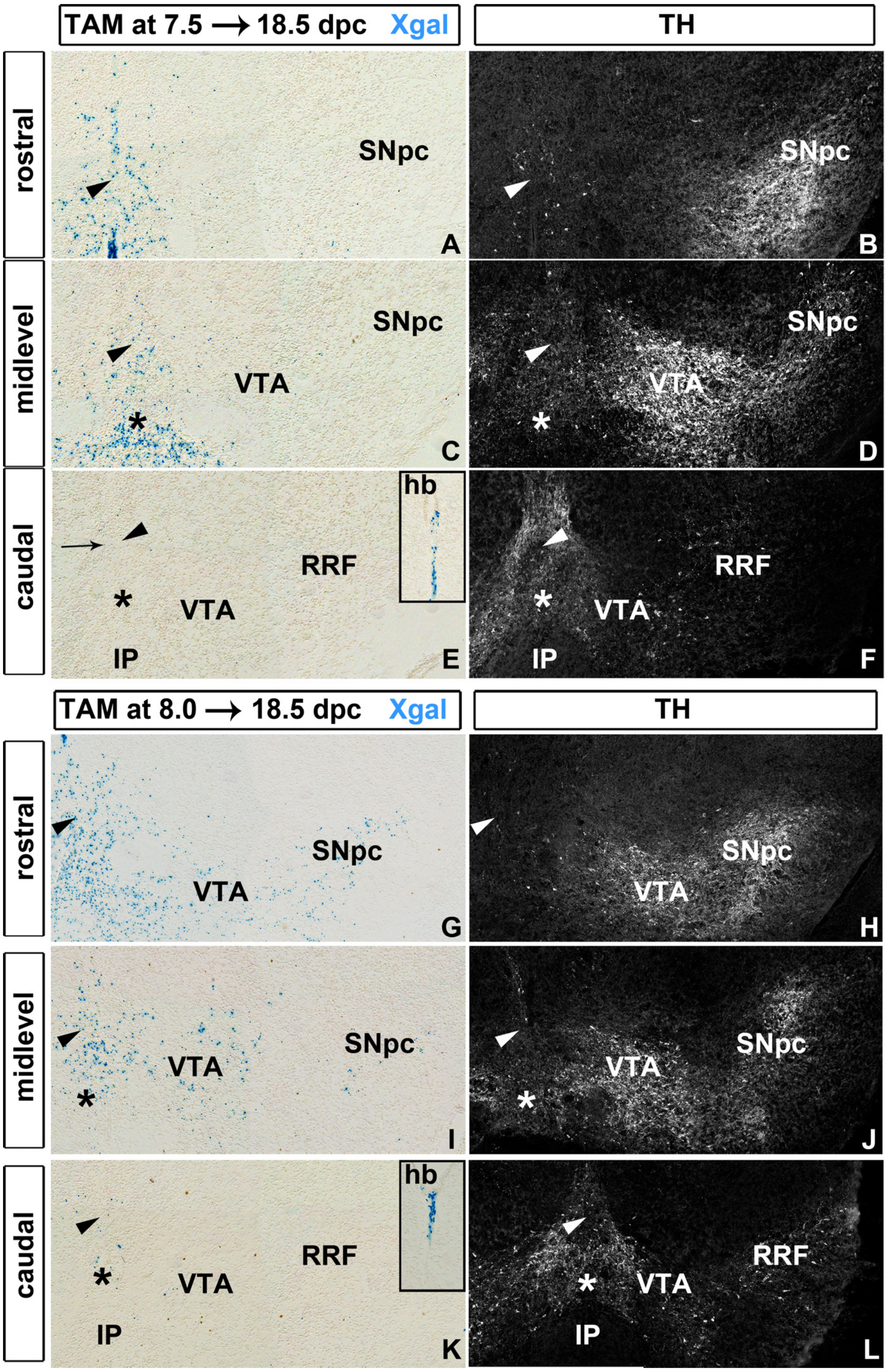

Fig. S4. Xgal and TH labeling on coronal midbrain sections along anterior posterior axes in 7.5 and 8.0 dpc tamoxifen-induced *Shh*::*CreER*^{T2},*R26R* embryos. (*A–F*) In 7.5 (injection) → 18.5 dpc (harvest), at rostral and midaxial levels, Xgal-labeled cells are observed predominantly in the rostral linear nucleus (arrowheads) and dopamine, (TH+), ventral tegmental area (VTA)/interfascicular nucleus (IF; asterisk) regions. [Note, the section in *D* (TH detection) is immediately rostral to the section in *C* (Xgal detection), and contains only the rostral tip of the IF with some TH+ cells (asterisk in *D*).] In contrast, very few Xgal+ cells are observed in the substantia nigra pars compacta (SNpc). At caudal levels, a few Xgal cells are detected in the rostral linear nucleus (arrow), but none in the VTA/IF (asterisk) regions or the retrorubral fields (RRF). The hindbrain midline shows several labeled cells (*E Inset*). (*G–L*) 8.0 (injection) → 18.5 dpc (harvest), at rostral and midaxial levels, results in labeling of cells in the rostral linear nucleus (arrowheads) and dopamine, (TH+), neurons in the VTA/IF (asterisk) regions as well as in the SNpc (*G* and *I*). At the caudal levels (*K*), some Xgal cells are detected in the rostral linear nucleus (arrowhead) and dopamine, (TH+), VTA/IF (asterisk) regions, but none in the RRF. Xgal cells are also observed in the hindbrain midline (*K Inset*). TH-labeled sections in *B*, *D*, *F*, *H*, *J*, and *L* were adjacent to the Xgal-labeled sections shown in *A*, *C*, *E*, *G*, *I*, and *K*, respectively. Adjacent to these were sections labeled with Pitx3 (not shown) used to determine the position of the predominantly TH–/Pitx3+ rostral linear nucleus. Taken together, these findings show that differential labeling of dopamine clusters along the anterior-posterior axis of the midbrain is dependent upon the time of TAM administration. IP, interpeduncular nucleus.

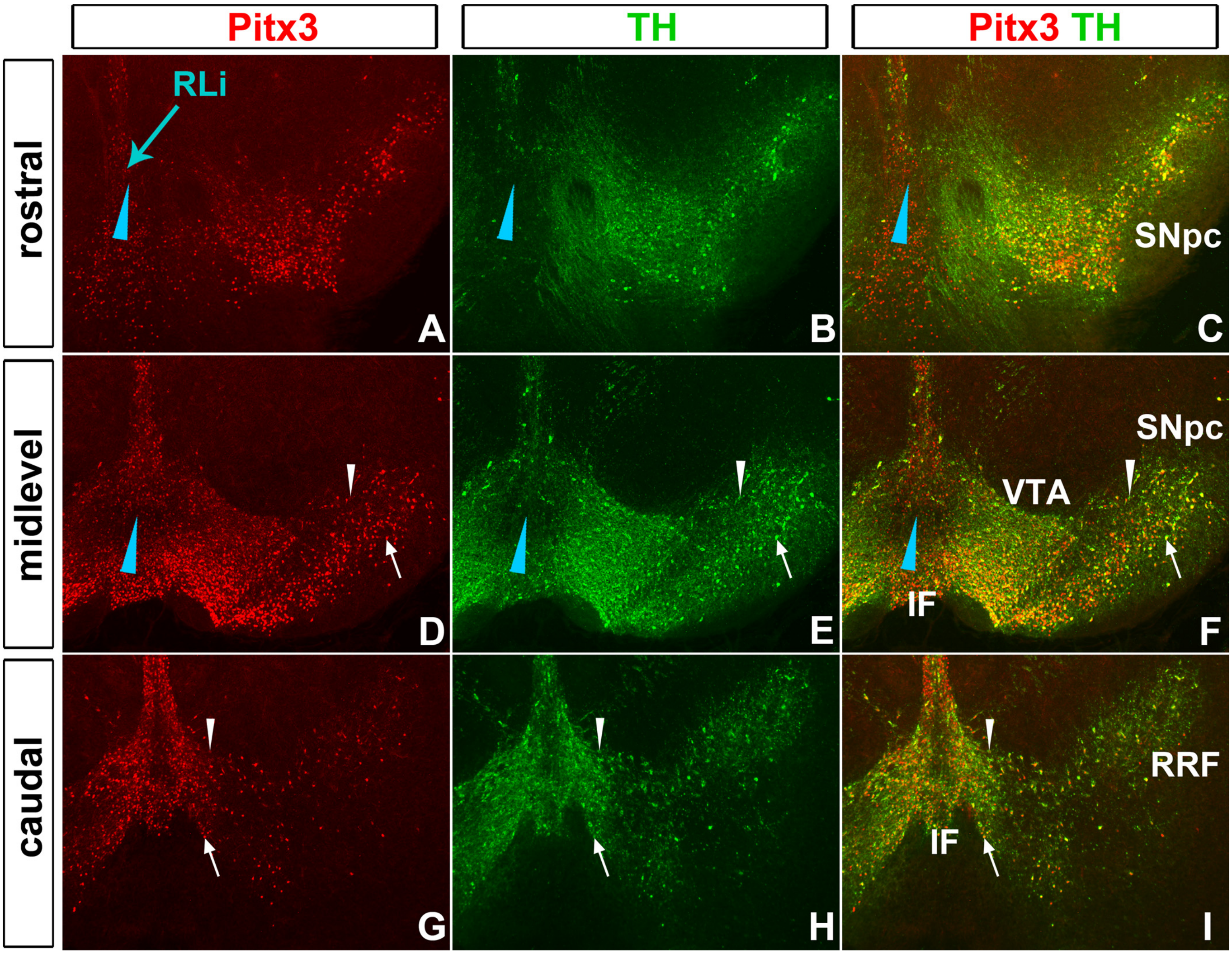

Fig. S5. Neurons in the rostral linear nucleus are Pitx3+ but show little to no TH immunoreactivity. (*A*–*I*) 18.5-dpc coronal sections labeled with Pitx3 (red) and TH (green). Pitx3+ neurons are detected in rostral linear nucleus (RLi; blue arrow in *A* and blue arrowheads); many of these neurons have little to no TH immunoreactivity. In contrast, neurons in the substantia nigra pars compacta (SNpc), ventral tegmental area (VTA)/interfascicular nucleus (IF) regions, and retrorubral fields (RRF) coexpress TH and Pitx3. Note also that dopamine (TH+) neurons expressing low (white arrowheads) and high (arrows) levels of Pitx3 are predominantly situated in the dorsal and ventral TH+ regions, respectively. Separate channels in (*A* and *B*), (*D* and *E*), and (*G* and *H*) are shown as merged images in (*C*), (*F*), and (*I*), respectively.

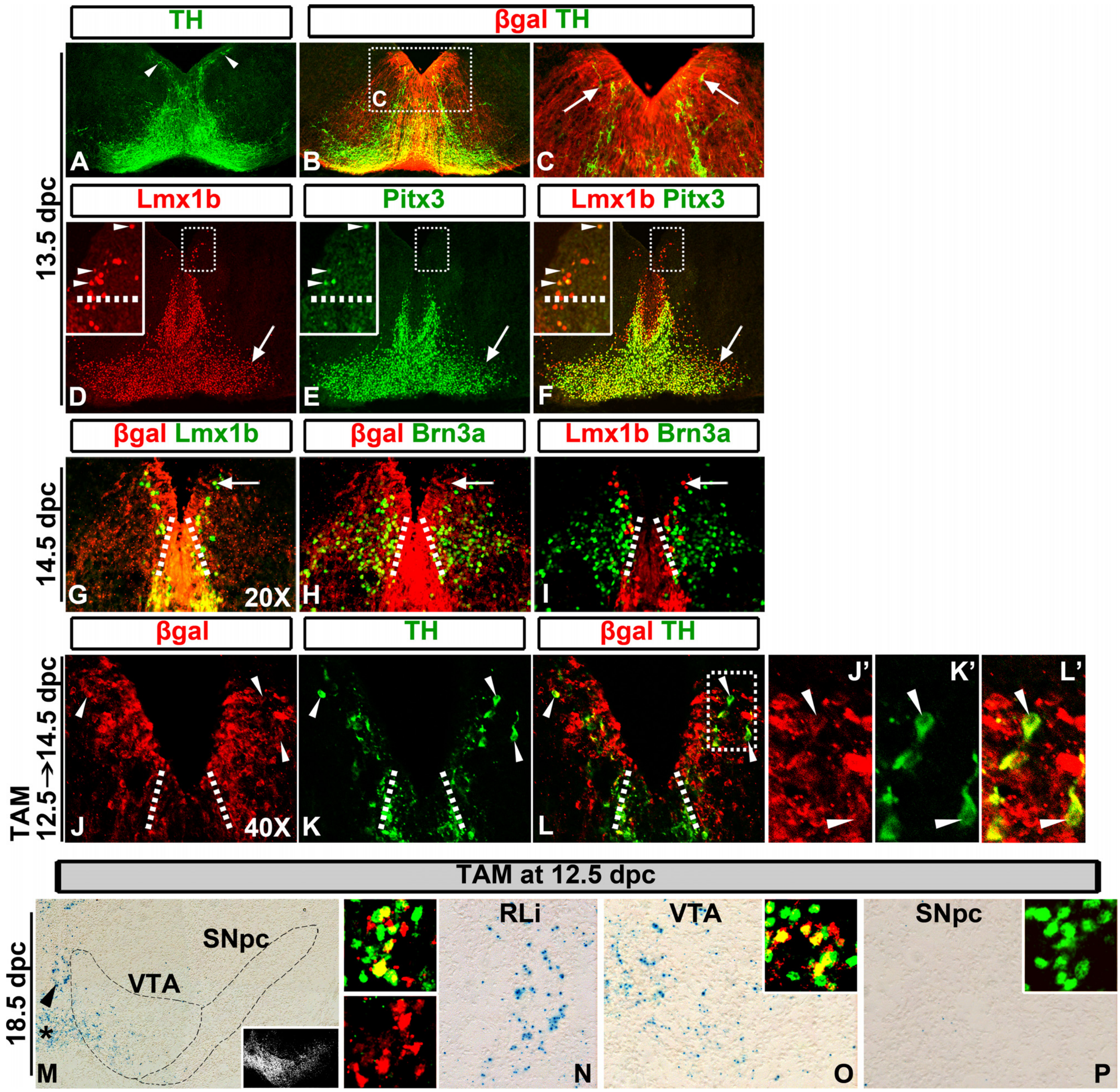

Fig. S6. Lateral *Shh* domain produces dopamine neurons. (*A*) A 13.5-dpc section shows TH+ neurons appearing to emerge from the lateral progenitors (arrowheads). (*B* and *C*) *Shh*::*Cre,R26R* sections show that some TH+ neurons (arrows) are observed emanating from the lateral β-gal domain. (*C*) High magnification of the region indicated by white dotted lines in (*B*). (*D*–*F*) Sections labeled with Lmx1b (red) and Pitx3 (green) at 13.5 dpc. Note that some Lmx1b+/Pitx3+ postmitotic neurons appear to emerge from the lateral, Lmx1b–, but not Lmx1b+ ventricular zone (separated by dashed lines). Arrowheads indicate neurons coexpressing Lmx1b and low levels of Pitx3. In addition, several neurons expressing low levels of Pitx3 (arrow) are situated in the dorsal regions of the mDA cohort. Separate channels in (*D*) and (*E*) are shown as the merged image in (*F*). Insets are high-power images of the regions indicated by dotted lines. (*G*–*I*) Triple immunofluorescence of β-gal (red in *G* and *H*), Lmx1b (green in *G*; red in *I*), and Brn3a (green in *H* and *I*) at 14.5 dpc further shows that Lmx1b+ postmitotic neurons (arrows) appear to originate from the lateral domain (dashed lines indicate the separation between Lmx1b+ and lateral, Lmx1b–, ventricular zone; see also Fig. S7). To label the lateral domain, TAM injections were performed at 11.5 and 12.5 dpc. In 11.5-dpc injections, in addition to lateral progenitors, significant labeling was observed in medial and intermediate domains, particularly caudally. Thus, we subsequently focused on 12.0- to 12.5-dpc injections. In *Shh*::*CreER*^{T2},*R26R* embryos, 12.5 (injection) → 14.5 dpc (harvest) lateral progenitors were preferentially labeled (*J*–*L*). Some β-gal+/TH+ neurons appear to emanate from these lateral β-gal+ progenitors (*J′*–*L′*). In *Shh*::*CreER*^{T2},*R26R* embryos, 12.5 (injection) → 18.5 dpc (harvest) (*M*–*P*) results in many Xgal-labeled cells in the RLi (arrowhead in *M*, and *N*) and VTA/IF (*M* and *O*), but few in the SNpc region (*M* and *P*). Thus, the lateral *Shh* domain does produce some dopamine neurons. (*N*–*P*) High-magnification images of the regions indicated by the arrowhead (RLi) and dotted lines (VTA/SNpc) in (*M*), respectively. Double labeling for β-gal (red) and Pitx3 (green; *Upper*) or TH (green; *N Lower Inset*) in the corresponding regions. (*M Inset*) TH immunoreactivity on the adjacent section relative to the corresponding Xgal labeling.

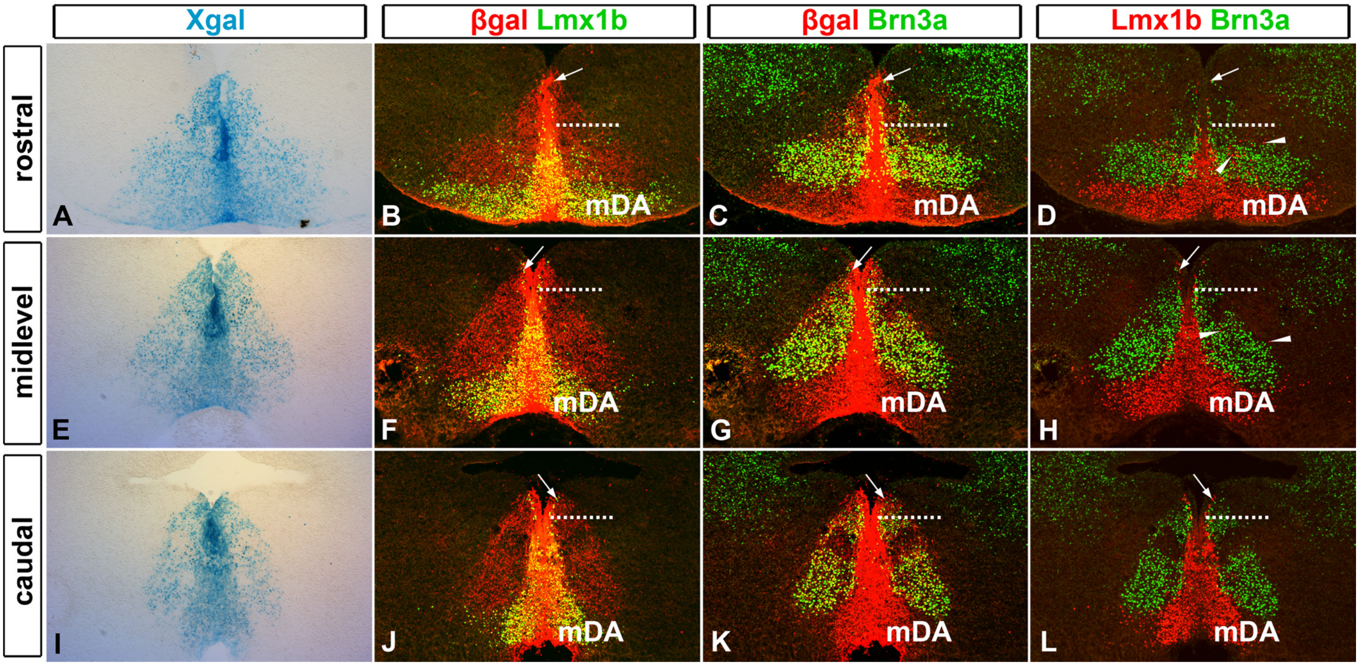

Fig. S7. A Brn3a cohort and some dopamine neurons appear to be produced from the lateral *Shh* domain in the developing midbrain. (*A*–*L*) Xgal (*A*, *E*, and *I*) and triple labeling with antibodies that recognize β-gal (Shh fate-mapped cells; red in *B*, *C*, *F*, *G*, *J*, and *K*), Lmx1b (green in *B*, *F*, and *J*; red in *D*, *H*, and *L*), and Brn3a (green in *C*, *D*, *G*, *H*, *K*, and *L*) on midbrain coronal sections of *Shh*::*Cre,R26R* embryos at 14.5 dpc. Note that Lmx1b+ neurons (white arrows) are detected within the lateral-most aspect of the *Shh* domain (β-gal+; *B*, *F*, and *J*). Likewise, Brn3a cells are also detected within the lateral aspect of the β-gal+ domain (*C*, *G*, and *K*). Some Lmx1b+ neurons (arrows) are detected well lateral to the Lmx1b+ ventricular zone (dashed lines indicate the separation between Lmx1b+ and lateral, Lmx1b– ventricular zone), and in close vicinity to Brn3a+ cells (*D*, *H*, and *L*). In addition, some Lmx1b+ neurons can be also seen within the Brn3a+ cohort, in the region of the nascent red nucleus (arrowheads). High-magnification images depicting laterally derived mDA are shown in Fig. S6 *G*–*I*. mDA, midbrain dopamine neurons.

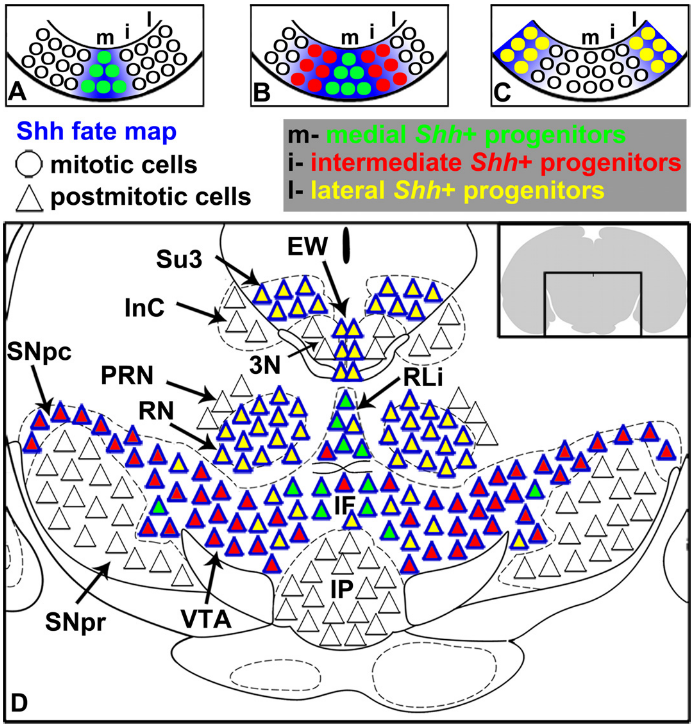

Fig. S8. A model for the embryonic origin of midbrain nuclei derived from spatiotemporally separable *Shh*+ progenitors (mid to caudal levels). (*A–C*) Schematic representation of *Shh*+ domains during midbrain development. Based upon cumulative and inducible genetic fate-mapping results, we estimate 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–). (*D*) Schematic of the adult organization of major midbrain nuclei with respect to their embryonic origin. The color within each triangle depicts the possible progenitor domain from which these nuclei are derived. The medial progenitors (green circles in *A* and *B*) predominantly contribute to the rostral linear nucleus (RLi; mostly TH–) and sparsely to the ventral tegmental area (VTA)/interfascicular nucleus (IF) regions, but remarkably little to the substantia nigra pars compacta (SNpc). The intermediate progenitors (red circles in *B*) contribute to the RLi and dopamine, TH+, neurons in the VTA/IF regions and SNpc. The lateral *Shh*+ progenitors (yellow circles in *C*) initially delineate the primordium of a Brn3a+ cohort that populates the Edinger Westphal nucleus (EW), supraoculomotor nucleus and cap (Su3), and the red nucleus (RN). The lateral progenitors also generate some TH+ neurons. Although in close proximity to Shh descendants (blue triangle contour) in the postnatal coronal midbrain sections, several cell populations are largely unlabeled (black triangle contour) in our experiments. Thus, these cells must be derived outside of *Shh*-expressing progenitors. These cells belong to the oculomotor nucleus (3N; Isl1/2+), interpeduncular nucleus (IP), and GABAergic neurons of the substantia nigra pars reticulata (SNpr), the pararubral nucleus (PRN), and the interstitial nucleus of Cajal (InC). Schematic of the midlevel midbrain coronal section is shown in (*D*) [adapted from Paxinos and coworkers' brain atlas (1)]. Midbrain structures were defined anatomically according to the mouse brain atlases (1, 2) and molecularly, if applicable, using a marker analysis (note: rostral sections shown in this article would be, strictly speaking, considered diencephalic rather than mesencephalic, according to refs. 3 and 4); in accordance with their terminology, we find that the dopaminergic primordium appears to extend from the isthmus to the zona limitans intrathalamica (Fig. 1*M*). We use ''midbrain dopamine neurons (mDA),'' as have others (5, 6), to avoid confusion with hypothalamic dopaminergic groups A11–14. Marker composition and anatomical location of the major ventral midbrain nuclei are as follows: (1) Rostral linear nucleus (RLi; Pitx3+, Lmx1a+*, Lmx1b+*, Nurr1+*, TH–) is located between mammillotegmental tracts; (2) interfascicular nucleus (IF; Pitx3+, Lmx1a+, Lmx1b+, Nurr1+, TH+) is located between the fasciculus retroflexus tracts and immediately ventral to the RLi at rostral levels; it is separated from the RLi by the ventral tegmental decussation at midlevels and caudal levels; (3) ventral tegmental area (VTA) and substantia nigra pars compacta (SNpc; Pitx3+, Lmx1a+, Lmx1b+, Nurr1+, TH+) are separated by the medial lemniscus at midlevels; (4) substantia nigra pars reticulata (SNpr; GATA3+) is located ventral to the SNpc; (5) subthalamic nucleus (Pitx3–, Lmx1a+, Lmx1b+, Nurr1–, TH–) has the ventrolateral position in the posterior hypothalamus immediately adjacent to the cerebral peduncle and rostral to the SNpc; (6) pararubral nucleus (PRN; GATA3+) is located immediately lateral to the RN; (7) nucleus of Darkschewitsch (Dk; GATA3+) is situated ventrolaterally in the periaqueductal gray just dorsal to the medial accessory nucleus (MA3); (8) interstitial nucleus of Cajal (InC; GATA3+) is abutting the periaqueductal gray just lateral to the Dk; (9) oculomotor nucleus (3N; Isl1/2+) is located lateral to the EW, dorsal to the RN, and ventral to the Su3; (10) Edinger Westphal nucleus (EW; Brn3a+) is a medial structure immediately dorsal to the RLi and flanked by the MA3 rostrally or the 3N caudally; (11) red nucleus (RN; Brn3a+) is located immediately dorsal to the VTA and lateral to the EW/RLi; and (12) supraoculomotor nucleus and cap (Su3; Brn3a+) is situated in proximity to the EW and immediately dorsal to the 3N. *Because Lmx1a, Lmx1b, and Nurr1 are also expressed in the posterior hypothalamus and mammillary body, Pitx3 immunoreactivity was used to distinguish these structures from the RLi.

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2. Paxinos G, Ashwell K, Tork I (1994) *Atlas of the Developing Rat Nervous System* (Academic, San Diego), 2nd Ed.
3. Smidt MP, Burbach JP (2007) How to make a mesodiencephalic dopaminergic neuron. *Nat Rev Neurosci* 8(1):21–32.
4. Verney C, Zecevic N, Puelles L (2001) Structure of longitudinal brain zones that provide the origin for the substantia nigra and ventral tegmental area in human embryos, as revealed by cytoarchitecture and tyrosine hydroxylase, calretinin, calbindin, and GABA immunoreactions. *J Comp Neurol* 429(1):22–44.
5. Ang SL (2006) Transcriptional control of midbrain dopaminergic neuron development. *Development* 133(18):3499–3506.
6. Prakash N, Wurst W (2006) Genetic networks controlling the development of midbrain dopaminergic neurons. *J Physiol* 575(Pt 2):403–410.

Table S1. Semiquantitative analysis of Xgal-labeled cells in Pitx3+ and Brn3a+ midbrain clusters at 18.5 dpc

TAM[a] at	SNpc	VTA/IF	RLi	RN	EW	Su3
7.5 dpc	±	+	+++	–	–	–
8.0 dpc	+++	+++	++++	±	–	–
8.5–9.5 dpc	++++	++++	++++	+++++	+++	+++
12.5	±	+	+	±	++	++

[a]TAM activity (and therefore corresponding recombinase activity) is maintained up to 36 h postinjection. (*Left*) In 7.5-dpc TAM injections, in which medial progenitors are labeled, fate-mapped cells are predominantly localized to the RLi, although some are visible in the VTA/IF; few fate-mapped cells are observed in the SNpc. In 8.0-dpc injections, medial and intermediate progenitors are labeled; fate-mapped cells are observed in the SNpc, VTA/IF, and RLi. In both 8.5- and 9.5-dpc injections, fate-mapped cells are observed in all the Pitx3+ clusters. In 12.5-dpc TAM injections, lateral progenitors are labeled and fate-mapped cells are observed in the RLi and VTA/IF region with a few if any in the SNpc. (*Right*) The Brn3a+ neuron cohort localizes mainly to the RN, EW, and Su3. In 7.5-dpc TAM injections, in which medial progenitors are labeled, no labeled cells are observed in these clusters. In 8.0-dpc injections, medial and intermediate progenitors are predominantly labeled, but few lateral progenitors are also labeled. Thus, occasional labeled cells are observed in these clusters. In both 8.5-dpc and 9.5-dpc injections, when medial, intermediate, and lateral domains are labeled, maximal labeling is observed in all the Brn3a+ clusters. In 12–5 dpc injections, lateral progenitors are preferentially labeled, and contribute to the EW and Su3 but less to the RN. This is in accordance with birthdating studies of the RN, showing a relatively early neurogenetic interval ending at 12.5 dpc. Together, these data suggest that the Brn3a cohort is derived from lateral *Shh*+ progenitors.