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Duration of Shh signaling contributes to mDA neuron diversity

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ABSTRACT

Sonic hedgehog (Shh) signaling is critical for various developmental processes including specification of the midbrain dopamine (mDA) neurons in the ventral mesencephalon (vMes). While the timing of *Shh* and its response gene *Gli1* segregates mDA neurons, their overall lineage contribution to mDA neurons heavily overlaps. Here, we demonstrate that the same set of mDA neuron progenitors sequentially respond to Shh signaling (*Gli1* expression), induce *Shh* expression, and then turn off Shh responsiveness. Thus, at any given developmental stage, cells rarely co-express *Shh* and *Gli1*. Using $Shh^{Cre:GFP}$ mice to delete the Smoothened receptor in the Shh pathway, we demonstrate that the loss of Shh signaling in *Shh* expressing cells results in a transient increase in proliferation and subsequent depletion of mDA neuron progenitors in the posterior vMes due to the facilitated cell cycle exit. Moreover, the change in duration of Shh signaling in vMes progenitors altered the timing of the contribution to the ventral tegmental area (VTA) and the substantia nigra pars compacta (SNc) mDA neurons. Taken together, our investigation on the relationship between the Shh-secreting and -responding cells revealed an intricate regulation of induction and cessation of Shh signaling that influences the distribution of mDA neurons in the VTA and SNc.

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Introduction

Sonic hedgehog (Shh) signaling plays essential roles in patterning and formation of many structures during development including the spinal cord, limb, and ventral mesencephalon (vMes) (Fuccillo et al., 2006; Ingham and Placzek, 2006; Chiang et al., 1996; Litingtung and Chiang, 2000; Zhang et al., 2001; Kraus et al., 2001; Bai et al., 2002; Wijgerde et al., 2002). Shh is a secreted molecule that diffuses away from the *Shh*-expressing cells. Upon Shh binding to the patched (Ptch1) receptor in Shh-responding cells, the Smoothened (Smo) receptor transduces intracellular signaling which converges on the Gli family of transcription factors (reviewed in Ingham and Placzek (2006)). Among the Glis, Gli2 functions primarily as a transcriptional activator that induces expression of many target genes, such as *Gli1*, which is used as an accurate and sensitive read-out for active Shh signaling in Shh-responding cells (Bai et al., 2002; Ahn and Joyner, 2004; Ahn and Joyner, 2005).

Various tissues, including the neural tube and limbs, are properly patterned and their cell types specified through the dynamic temporal and spatial control of *Shh* expression and responsiveness during development. Interestingly, Shh-responsiveness is necessary and sufficient for induction of Shh ligand expression (Matise et al., 1998; Ye et al., 1998). Thus, the tight regulation of Shh responsiveness is controlled by Shh ligand expression and the ability of the receiving cells to transduce the Shh signal.

The vMes is an ideal model for studying the dynamic nature of Shh signaling because *Shh* expression and Shh-responsiveness (*Gli1* expression) are temporally and spatially regulated during vMes development (Hayes et al., 2011; Zervas et al., 2004; Blaess et al., 2006; Joksimovic et al., 2009a). Dynamic changes in *Shh* and *Gli1* expression in the vMes are translated into a distinct contribution pattern of midbrain dopamine (mDA) neurons (Hayes et al., 2011; Blaess et al., 2011; Joksimovic et al., 2009a), which are subdivided into the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc) mDA neurons based on their anatomical location (Van den Heuvel and Pasterkamp, 2008). Interestingly, *Gli1* expression is rapidly downregulated in Shh responding cells after induction of the Shh ligand (Hayes et al., 2011). This raises the possibility that the duration of Shh signaling in vMes progenitors may differ as some progenitors become refractory and lose their ability to respond to Shh signaling. In the developing limb and neural tube, changes in the duration of active Shh signaling determine digit identity (Zhu et al., 2008) and ventral neuronal cell types (Ribes et al., 2010), respectively. However, whether a similar mechanism contributes to mDA neuronal subtype development has not been addressed.

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In this study, we manipulated the timing and duration of Shh signaling in the vMes and assessed the development of mDA neurons. Our comprehensive comparison of the expression pattern and short term genetic lineage analysis of *Shh* and *Gli1* revealed a unique relationship in which *Shh* expression is induced in the Shh-responding cells. Furthermore, our genetic manipulations, which alter the timing and duration of Shh signaling by removing the Shh signaling receptor, *Smo*, in *Shh*-expressing cells, revealed a functional role for the tight temporal regulation of Shh signaling. Together, these studies demonstrate a functional requirement for dynamic Shh signaling in regulating the cell cycle status of mDA progenitors to ultimately influence their final distribution in the VTA and SNc.

Materials and methods

Animals

Mouse lines were maintained on an outbred Swiss Webster background. See Supplementary Table 1 for a description of each mouse allele. For breeding, male $Shh^{Cre:GFP/+}$ mice were crossed with $Gli1^{nLacZ/+}$ females to generate $Shh^{Cre:GFP/+}$;$Gli1^{nLacZ/+}$ embryos. Additionally, male $Gli1^{CreER/+}$;$R26^{tdTomato/tdTomato}$ mice were crossed with wildtype or $Gli1^{nLacZ/+}$ females to generate $Gli1^{CreER/+}$;$R26^{tdTomato/+}$ and $Gli1^{CreER/nLacZ}$;$R26^{tdTomato/+}$ embryos, respectively. For *Smo* loss of function experiments, male $Shh^{Cre:GFP/+}$;$R26^{YFP/YFP}$ mice were crossed with wildtype females to generate $Shh^{Cre:GFP/+}$;$R26^{YFP/+}$ control embryos. For mutant embryos, male $Shh^{Cre:GFP/+}$;$Smo^{+/-}$ mice were crossed with $Smo^{Flox/Flox}$;$R26^{YFP/YFP}$ females to generate $Shh^{Cre:GFP/+}$;$Smo^{Flox/-}$;$R26^{YFP/+}$ mutant embryos. All animals were housed and handled according to the National Institutes of Health Institutional Animal Care and Use Committee guidelines.

Tamoxifen, EdU, and BrdU injections

Briefly, between 9:00 and 10:00 a.m. on E7.5, E8.5, or E9.5, 2 mg of tamoxifen (TM) was delivered by oral gavage to the timed–pregnant dams using a disposable feeding needle (FST 9921) (Brown et al., 2009).

EdU (5-ethynyl-2′-deoxyuridine, Invitrogen, A10044) and BrdU (5-bromo-2′-deoxyuridine, Invitrogen, B23151) were prepared as 2.5 μg/μl and 10 μg/μl stock solutions, respectively, in sterile PBS and stored at −20 °C. EdU or BrdU was warmed to 37 °C and delivered by intraperitoneal injection to the pregnant dams in the evening of E10.5, 11.5 or E13.5 at a dose of 20.8 mg/kg of body weight for EdU and 200 mg/kg of body weight for BrdU. Animals were sacrificed 1 h after injection for proliferation analysis and at E13.5 for cell cycle exit study.

Tissue processing

The collection and processing of tissues were as described (Hayes et al., 2011). Briefly, tissue was fixed in 4% paraformaldehyde (PFA) overnight, rinsed in PBS, cryoprotected in a sucrose gradient, embedded in optimal cutting temperature (OCT), frozen in liquid nitrogen-chilled isopentane, and sectioned on the Leica Cryostat (CM3050S) (Brown et al., 2009). Sections were collected at 10 μm (E10.5), 12 μm (E11.5 and E13.5), and 14 μm (E16.5) and stored at −80 °C.

RNA in situ hybridization

Shh and *Gli1* probes were described previously (Platt et al., 1997). RNA in situ hybridization was performed essentially as described (Blaess et al., 2006). The hybridized RNA in situ probe was detected within 6 h for *Shh* and 24 h for *Gli1*. The developed sections were washed with PBS, fixed in 4% PFA, washed with PBS again, and coverslipped with Fluoromount-G (SouthernBiotech) mounting media.

Fluorescent Immunohistochemistry and X-gal histochemistry

Immunohistochemistry was performed as described (Hayes et al., 2011; Wang et al., 2011). The antibodies used are listed in Supplemental Table 2. EdU detection was performed using the Click-iT EdU Imaging Kit (Invitrogen, C10340) according to the manufacture's guidelines. Briefly, after incubation in secondary antibodies and washing in 0.2% TritonX-100/PBS (PBT), the sections were incubated in EdU staining solution (1X Click-iT reaction buffer, $CuSO_4$, Alexa 647, 1X reaction buffer additive) for 30 min in a dark humid chamber, washed in PBT several times, counterstained in Hoescht (Invitrogen, H3569), washed in PBS, and coverslipped with Fluoromount-G mounting media. X-gal histochemistry to detect *LacZ* expression was performed as described (Hayes et al., 2011; Ahn and Joyner, 2004).

Microscopy

All fluorescent images were captured using a Leica DM6000 upright microscope equipped with a Hamamatsu ORCA-ER digital camera and the Volocity software (PerkinElmer) or Zeiss Axiovert 200M microscope with LSM510 Meta confocal system. Bright field images were captured with a MacroFire (Optronics) digital camera and PictureFrame (Optronics) software. Images were processed with Photoshop in Adobe Creative Suite 3 (San Jose, CA) for brightness and contrast levels.

Quantification and statistical analyses

At E10.5, 11.5, and E13.5, the Lmx1a and EdU co-staining was quantified using the measurement function in Volocity (PerkinElmer). Lmx1a and EdU staining was quantified by measuring the area that contained pixels with intensity greater than 1 standard deviation from the peak of the pixel intensity distribution. Lmx1a and EdU are both nuclear stains, which allowed Volocity to measure the area of their co-expression. We then calculated the percentage of proliferating Lmx1a area by dividing EdU and Lmx1a double positive area by total Lmx1a area ($Lmx1a^+$ EdU^+/Total $Lmx1a^+$). Coronal sections were matched for anterior/posterior level based on the distribution pattern of TH^+ or $Lmx1a^+$ cells.

At E16.5, stereological counting was performed on 14 μm thick samples collected in 8 sets. Using the StereoInvestigator System (MicroBright Field, Inc.), the SNc and VTA mDA neuron area was outlined, and 40 μm by 40 μm counting frames were systematically placed every 100 μm by 100 μm over the outlined area. The number of TH^+ EdU^+ cells and only TH^+ cells were counted in each counting frame. The percentage of mDA neurons derived from progenitors that were proliferating at E11.5 or E13.5 were determined by the ratio of TH^+ EdU^+ co-expressing cells counted to total TH^+ cells counted (TH^+ EdU^+/Total TH^+) for each area (SNc, VTA, and total mDA neurons). Number of brains/embryos used is indicated as n in the Results section and the quantified data are presented as the mean value ± the standard error of means (s.e.m). The statistical analyses were performed using the Student's *t*-test and $p \leq 0.05$ was considered statistically significant.