Dynamic temporal requirement of Wnt1 in midbrain dopamine neuron development

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SUMMARY

Wnt1-expressing progenitors generate midbrain dopamine (MbDA) and cerebellum (Cb) neurons in distinct temporal windows and from spatially discrete progenitor domains. It has been shown that Wnt1 and Lmx1a participate in a cross-regulatory loop that is utilized during MbDA neuron development. However, Wnt1 expression dynamically changes over time and precedes that of Lmx1a. The spatial and temporal requirements of Wnt1 in development and specifically its requirement for MbDA neurons remain to be determined. To address these issues, we generated a conditional Wnt1 allele and temporally deleted Wnt1 coupled with genetic lineage analysis. Using this approach, we show that patterning of the midbrain (Mb) and Cb by Wnt1 is determined. To address these issues, we generated a conditional Wnt1 allele and temporally deleted Wnt1 coupled with genetic lineage analysis. Analysis of this approach, we show that patterning of the midbrain (Mb) and Cb by Wnt1 is determined. 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However, all WNTs that use canonical signaling converge on β-catenin, which precludes an understanding of the specificity of WNT ligand involvement in MbDA neuron development in vivo. Genetic inducible fate mapping (GIFM) shows that distinct spatial and temporal epochs underpin Wnt1 lineage contribution to MbDA neurons and to Cb neurons (Brown et al., 2011; Hagan and Zervas, 2012). Furthermore, dynamic oscillatory genetic networks in human neuronal progenitors are influenced by WNT1 (Wexler et al., 2011). However, the functional requirement of WNT1 in developmental processes is unresolved. Thus, we generated a conditional knockout allele of Wnt1 that we coupled with GIFM to uncover distinct spatial and temporal requirements for Wnt1 in patterning the Mb and Cb, controlling ventral mesencephalon (v.Mes) progenitors and regulating cell cycle exit during MbDA neuron development.

MATERIALS AND METHODS

Wnt1 conditional knockout targeting construct (pWnt1neoflox)

Wnt1 conditional knockout constructs were generated by recombinecing (supplementary material Fig. S1) (Liu et al., 2003). Briefly, we captured Wnt1 and modified it by placing a loxP-PGK-gb2-neo-loxP cassette between the third and fourth exons (LoxP) (supplementary material Fig. S1A,B, Table S1). Recombinants were determined by positive selection followed by pKS-Cre removal of the Neo cassette. A second FRT-PGK-gb2-neo-FRT-loxP cassette was inserted between the first and second exons (LoxP-FRT; Table 1) and was used for selection in embryonic stem (ES) cells. The targeting vector was designated as pWnt1neoflox (supplementary material Fig. S1B). Plasmids were verified by PCR, restriction digestion and sequencing. The recombination potential of pWnt1neoflox was determined by expressing it in Escherichia coli cells electropoerated with pKS-Cre, resulting in the deletion of exons 2 and 3 (supplementary material Fig. S1C).

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Gene targeting and conditional gene manipulations

JMJ6.6 E5 ES cells of C57Bl/6 origin (generously provided by Dr Bill Skarnes) (Pettit et al., 2009) were electroporated with 20 μg of linearized pWnt1neoac. G418-resistant clones were confirmed by PCR screening for the Neo cassette (primers 3, 5) and positive clones were screened for the absence of the AMP cassette by PCR (primers 9, 10). Clones were also screened for the downstream loxP site (primers 1, 2). Primer sequences are provided in supplemental material Table S2. Two correctly targeted ES clones, PL1G0 and PL1G12, were karyotyped as 95% normal and used for blastocyst injection. Correctly targeted Wnt1neoac founders were derived from germ line chimera and bred to F0 mice (The Jackson Laboratory #003946) to delete the Neo cassette. Subsequent matings eliminated the F0 allele to generate the Wnt1neoac/neo (Wnt1neoac mice were bred with the following lines: (1) Enf1Cre for early [starting at embryonic day (E) 8.0] cumulative recombination (Kimmel et al., 2000; Chi et al., 2003; Ellison et al., 2009); (2) Gbx2fl/fl;cre;R26Ltmom (Chen et al., 2009; Luu et al., 2011) and (3) Wnt1-CreER to tamoxifen for temporal control of recombination (Zervas et al., 2004; Brown et al., 2011); (4) ShhCre for cumulative recombination starting at E9.0-9.5 (Harfe et al., 2004; Hayes et al., 2011). Tamoxifen takes 6 hours to initiate recombination and lasts for 24-30 hours (reviewed by Ellison et al., 2009; Brown et al. 2009). Genotyping was carried out as previously described (Ellison et al., 2009). Rosa26fl-stop-flac-ltmom (R26Ltmom) mice were used for lineage tracing and Wnt1-Neomice were used to detect Wnt1 expression (Madisen et al., 2010; Brown et al., 2011; Ellison et al., 2012). All Cre and reporter lines in this study have been validated and described elsewhere (Kimmel et al., 2000; Li et al., 2002; Zervas et al., 2004; Madisen et al., 2010; Brown et al., 2011; Hagan and Zervas, 2012; Hayes et al., 2011). Mice were housed and handled in accordance with Brown University Institutional Animal Care and Use Committee guidelines.

In situ hybridization, β-galactosidase (β-gal) histochemistry and immunofluorescence immunocytochemistry

Full details of these experimental protocols have been previously published (Ellison et al., 2009; Brown et al., 2011; Hagan and Zervas, 2012). Primary antibodies for marker analysis were: anti-tyrosine hydroxylase (TH; Chemicon, 1:500), anti-5-hydroxytryptamine (5-HT; Jackson ImmunoResearch, 1:500), anti-OTX2 (Abcam, 1:250), anti-NURR1 (Santa Cruz, 1:200), anti-LMX1a (Michael German, UCSF, 1:1000), anti-GFP (Nacalai Tesque, 1:500) and anti-disred (1:500, Clontech). Secondary antibodies (Molecular Probes, 1:500) were: donkey anti-rabbit Alexa 555, donkey anti-rat Alexa 488, donkey anti-rabbit IgG-Alexa488, donkey anti-goat IgG-Alexa488, donkey anti-rabbit IgG-Alexa 350.

Cell cycle analysis

Pregnant dams were given 20 mg/kg 5-ethyl-2′-deoxyuridine (EdU) by intraperitoneal injection (Wang et al., 2011) and sections were labeled for Ki67 (1:100; rat monoclonal IgG2a; Dako, cat #M7294), TH (1:500; rabbit polyclonal IgG; Millipore-Chemicon, cat #AB152), RFP (1:1000; chicken polyclonal IgG; VWR, cat #RL600-901-379), EdU (Click-IT EdU Alexa Fluor 647 Imaging Kit; Invitrogen, cat #I30340) and Hoechst. EdU staining was carried out following the manufacturer’s instructions. Antigen retrieval was carried out for Ki67 immunolabeling by boiling sections in R-Buffer A (Electron Microscopy Sciences) and neutralizing with 0.1 M borate buffer.

Cell counts from animals of matched genotypes were summed (total count) and the total counts for each animal were averaged. A two-tailed t-test was used to analyze cell counts and to determine statistical significance (P-values are indicated in text).

Microdissection and fluorescence-activated cell sorting (FACS)

The v.Mes of Wnt1-Venus embryos was microdissected as previously described (Brown et al., 2009; Ellison et al., 2012). Briefly, we isolated the v.Mes under a stereomicroscope fluorescence microscope. The small domain of YFP-expressing cells was carefully dissected away from the posterior mes ‘ring’ and dorsal tissue. In addition, d.Mes or whole head was isolated. The isolated tissues were trypsinized using TrypLE Express (Gibco) and DNase (Roche) at 1:1000 for 8 minutes at 37°C. Subsequently, 10% PBS in PBS was added to stop the reaction. Samples were then mechanically dissociated using a 20-gauge needle and 1cc syringe to generate a single cell suspension. Cells were stored on ice until sorting with a FACS/Aria flow cytometer (BD Biosciences) and analyzed using Diva software (BD Biosciences) at Brown University’s Flow Cytometry and Sorting Facility. Population gates were established by setting threshold values based on wild-type littermate control samples and negative control samples obtained from the prosen cephalic. Cells were sorted into clean epiblasts containing a small volume of 10% PBS in PBS and spun at 3000 rpm (800 g) for 10 minutes at 4°C. PBS was removed and 100 µl of Trizol (Invitrogen) was added and samples were stored at –80°C until RNA extraction.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Cells in Trizol were thawed on ice and allowed to sit at room temperature for 5 minutes. Chloroform (25 µl) was added to each tube and shaken vigorously for 15 seconds and incubated at room temperature for 3 minutes. Samples were centrifuged at 4°C for 15 minutes. RNA was transferred to a new tube and isopropanol (50 µl) was added and incubated for 10 minutes at room temperature. Samples were spun at 13,200 rpm (16,100 g) for 15 minutes at 4°C. The RNA pellet was washed with 250 µl of 75% ethanol, spun at 7500 rpm (5100 g) for 5 minutes at 4°C, and resuspended in 20 µl of dH2O. RNA was quantified with a nanodrop and assed for purity with an Agilent Bioanalyzer. cDNA was synthesized using iScript cDNA Synthesis Kit (BioRad #170-8890) according to manufacturer’s instructions. We used 0.5 µg of RNA template, 4 µl of 5X iScript reaction mix, 1 µl of iScript reverse transcriptase (RT) in a 15 µl reaction. The thermocycle program was 25°C for 5 minutes, 42°C for 30 minutes, 85°C for 5 minutes. qRT-PCR was performed with SYBR Green (Applied Biosystems #4367659) in MicroAmp 96-well reaction plates. For each primer set, we used a reaction mix of nuclease-free water (7.5 µl), Primer 1 (2.0 µl), Primer 2 (2.0 µl), SYBR Green (12.5 µl). Note that all primers were tested to ensure that they yielded a single amplicon of appropriate size (typically ~200 nucleotides). All samples were processed in triplicate and dissociation curves were compared with those obtained for 18S. Relative RNA levels (averages±s.d.) were plotted using Numbers; statistical significance was determined using Student’s t-test (P<0.05). Primer sequences are provided in supplementary material Table S3.
RESULTS

Dynamic expression of Wnt1 in MbDA neuron progenitors

We examined Wnt1 at well-defined stages of MbDA neuron development (Ang, 2006) and focused on v.Mes progenitors because they are a substantial source of MbDA neurons in vivo (Brown et al., 2011). We used a genetics-based approach that has been instructive in assessing dynamic gene regulation in spinal cord (Luu et al., 2011). Specifically, we administered tamoxifen at E9.5 to pregnant females harboring Wnt1-CreER;R26tdTomato;Wnt1-Venus embryos followed by analysis 48 hours later (Fig. 1). In this short-term GFM experiment, the Wnt1-CreER;R26tdTomato allelic combination plus tamoxifen marked the Wnt1-expressing progenitor pool at E9.5 whereas the Wnt1-Venus transgene showed ‘current’ Wnt1 expression at the stage of analysis (E11.5). Wnt1-CreER;R26tdTomato;Wnt1-Venus embryos had Wnt1 lineage-derived cells (red fluorescence”) that were GFP” in the dorsal mesencephalon (d.Mes), indicating that Wnt1 expression in these progenitors had ceased over the 2 days following marking (Fig. 1A-C). By contrast, the Wnt1 lineage in the v.Mes appeared to be double positive (red fluorescence+/GFP”), suggesting that Wnt1 expression in v.Mes progenitors persisted (Fig. 1A-C). We analyzed the v.Mes by double immunolabeling with an anti-dsred antibody to identify the Wnt1 lineage (dsred”, red) and an anti-GFP antibody to identify Wnt1-expressing progenitors (GFP”, green) (Fig. 1D,E). In medial sections, much of the Wnt1 lineage marked at E9.5 continued to express Wnt1 (dsred”/GFP”, yellow cells) (Fig. 1D). However, off-midline sections showed heterogeneity of progenitors: Wnt1-lineage derived cells closest to the v.Mes flexure were dsred”/GFP” (Fig. 1E, yellow cells) interspersed amongst cells expressing GFP only (Fig. 1E, white arrowheads). Cells proximal to the ventricle were dsred” (Fig. 1E, purple arrowheads). Interestingly, MbDA neurons (tyrosine hydroxylase, TH”) at E11.5 were located in the differentiated zone at the v.Mes flexure and were derived from the Wnt1 lineage and continued to express Wnt1 (Fig. 1F; TH”/dsred”/GFP”, blue arrowheads). In addition, we observed clonal-like cohorts of cells that had expressed Wnt1 at E9.5 but had ceased to express Wnt1 by E11.5 (Fig. 1F,G; dsred”/GFP”, purple arrowheads), and clones that appeared to newly express Wnt1 (Fig. 1F,G; dsred”/GFP”, white arrowheads). One caveat of this last group is that some cells may have escaped marking owing to the mosaic nature of CreER-tamoxifen.

We observed a similar dynamic regulation when we administered tamoxifen at E8.5 and analyzed E12.5 embryos, with the exception of the marked Wnt1 lineage being more broadly distributed throughout the mes (data not shown). Thus, there was a cohort of the Wnt1 lineage that expressed Wnt1 early and then extinguished Wnt1 over the following 2-4 days and cells that expressed Wnt1 early and continued to express Wnt1. These findings showed dynamic regulation of Wnt1 and suggested distinct temporal roles for Wnt1 in development.

Generation of a conditional knockout allele of Wnt1

We generated a conditional knockout allele of Wnt1 using recombinant technology (Liu et al., 2003) to address the temporal requirement of Wnt1 in development (supplementary material Fig. S1). The entire Wnt1 gene was captured from a bacterial artificial chromosome (BAC) library (supplementary material Fig. S1A) and modified by cloning a loxP site between the third and fourth exons and cloning a FRT-PGK-gb2-neo-FRT-loxP cassette between the first and second exons (Fig. 2A; supplementary material Fig. S1B). Linearized Wnt1flox/+ was electroporated into mouse ES cells and clones that had undergone homologous recombination were used to generate chimera (Fig. 2A,B). Wnt1flox/+ founders were bred to Flpe mice to remove the Neo cassette (Fig. 2A,B). To validate the ability to convert the Wnt1flox/+ to the deleted allele in vivo (designated Wnt1-∆MHB), we bred Wnt1-∆MHB males with En1Cre females and analyzed the rostral and caudal halves of embryos at the 6- to 8-somite stage (n=3) by PCR, which confirmed the Wnt1-∆MHB allele in embryos that inherited En1Cre (supplementary material Fig. S2).

Conditional Wnt1 deletion disrupts mesencephalon and rhombomere 1 patterning

We generated and bred En1Cre;Wnt1flox/+ to Wnt1flox/+ mice to conditionally delete Wnt1 early in midbrain-hindbrain (MHB) development, consistent with En1 expression and En1Cre-mediated recombination (Li et al., 2002; Chi et al., 2003; Ellison et al., 2009). Operationally and for clarity of discussing the multiple Cre lines used in this study, we designated the deleted allele in the MHB as Wnt1MHBflox/+. Wnt1flox/+ and Wnt1-∆/+ embryos were morphologically normal at E10.5 (n=4) (Fig. 2D; supplementary material Fig. S3). By contrast, En1Cre;Wnt1MHB deleted conditional mutant littermates (n=3) had a severe deletion of the putative mes and rhombomere 1 (r1) (Fig. 2F; supplementary material Fig. S3). We assayed sagittal sections from controls and conditional mutants by molecular
marker analysis. In controls, Otx2, Pax6, Wnt1 and Fgf8 were expressed in well-defined domains at E10.5, as previously described (Fig. 2E-H; supplementary material Fig. S4A-G). En1Cre;Wnt1ΔMHB/+ mutants at E10.5 had Otx2 expression throughout the prosencephalon and diencephalon but not in the hypothalamus (Fig. 2J), which was similar to controls. Otx2 was also expressed in the remaining putative mes (Fig. 2J, red arrow) and r1 (Fig. 2J, red arrowhead) based on morphology. Pax6 delineated the diencephalon, but was not expressed in the remaining putative mes and r1 (Fig. 2K, red arrow and arrowhead, respectively) of conditional mutants. Wnt1 was not detected in the putative mes, but a small domain was observed in lateral r1 (Fig. 2L, red arrow and arrowhead, respectively) of conditional mutants. The posterior hindbrain (Hb) of En1Cre;Wnt1ΔMHB/+ embryos was unaffected because it was outside of the En1 domain (Fig. 2L). The presence of Wnt1 in a small lateral r1 domain suggested that En1Cre did not mediate recombination in this territory or that Wnt1 and En1 did not overlap in this region. Finally, conditional deletion of Wnt1 resulted in the absence of Fgf8 expression in the isthmus (anterior r1) (Fig. 2M). The v.Mes was dysmorphic in mutants and the small portion of the mutant v.Mes flexure was Otx2+/Pax6– (Fig. 2J,K versus 2E,F, black arrows; supplementary material Fig. S4).

Early requirement of Wnt1 in the En1 lineage

The molecular identity of conditional mutants suggested that some mes/r1 tissue persisted, which prompted us to assess the En1 lineage in Wnt1 conditional knockouts by coupling gene deletion and cumulative lineage marking. We compared En1Cre;Wnt1ΔMHB/+;R26Tdtametes control and En1Cre;Wnt1ΔMHB/ΔMHB,R26Tdtametes mutant littermates at E12.5 (n≥3 each genotype). Whole-mount analysis of controls showed the typical distribution of the En1 lineage (Fig. 3A-C, red fluorescence). By contrast, En1Cre;Wnt1ΔMHB/ΔMHB,R26Tdtametes mutant embryos had a substantial depletion of the En1 lineage-derived mes (Fig. 3H-K). In addition, r1 was diminished and had only a small domain that was adjacent to axonal bundles connected to the trigeminal ganglia (Fig. 3I). Given that the v.Mes and ventral r1 (v.r1) are where MBDA and serotonin neurons are generated, respectively, we assessed En1 lineage contribution with biomarkers of neural progenitors and differentiating neurons in these domains (as shown in Fig. 3C,K). The limits of the En1 lineage delineated the anterior extent of the mes and the posterior limit of r1 in control embryos consistent with previous reports (Fig. 3D-H, arrowheads) (Zervas et al., 2004; Ellisor et al., 2009). Otx2, which plays a role in MBDA neuron development (Di Salvio et al., 2010; Omodei et al., 2008; Vernay et al., 2005) was distributed within the En1-derived domain.
and had a sharp caudal limit at the mes/r1 boundary (Fig. 3D, arrows; supplementary material Fig. S5). LMX1a, which is a transcription factor proposed to be a determinant of MbDA neurons (Andersson et al., 2006), was robustly expressed in the ventricular zone and to a lesser extent in the differentiated zone adjacent to the v.Mes flexure with a caudal limit at the v.Mes/v.r1 boundary (Fig. 3E, arrows; supplementary material Fig. S5). LMX1a was also expressed in the ventral diencephalon (Fig. 3E). Tyrosine hydroxylase (TH)-expressing MbDA neurons were located in the differentiated zone of the En1-derived v.Mes of controls (Fig. 3F; supplementary material Fig. S5) whereas the cell bodies of 5-hydroxytryptamine (5-HT)-expressing serotonergic neurons were expressed in the ventral diencephalon (Fig. 3E). Tyrosine hydroxylase (TH)-expressing MbDA neurons were located in the differentiated zone of the En1-derived v.Mes of controls (Fig. 3F; supplementary material Fig. S5). LMX1a, which is a transcription factor proposed to be a determinant of MbDA neurons (Andersson et al., 2006), was robustly expressed in the ventricular zone (vz, yellow bracket) and had a sharp caudal limit at the mes/r1 boundary (Fig. 3D, arrows; supplementary material Fig. S5). LMX1a+ progenitors were absent in the remaining En1-derived ventral tissue (not shown), although more caudally positioned 5-HT+ neurons as indicated in text. (F) MbDA neurons in the differentiated zone (d2, yellow bracket); inset shows TH+ neurons at higher magnification. (G,H) 5-HT+ neurons in v.r1. Numbers indicate rostral to caudal 5-HT+ neurons as indicated in text. (I-P) En1<sup>fl/fl</sup>;Wnt1<sup>cre/+;R26tdTomato</sup> mutants at E12.5 (IJ). Wnt1 mutants were devoid of the mes (indicated by red arrow), but did have a small fused mes/r1 domain (mes/r1*) and sparse projections at the pontine flexure (yellow arrow). (K) Region of analysis and En1-lineage (as described for C). (L-P) The En1-derived mes/r1 domain (red) was diminished, but a cohort of En1 lineage-derived cells was present. The anterior boundary was irregular and dsRed<sup>+</sup> cells were in the dl. (LM) Wnt1-mutant/En1-lineage derived domain had OTX2 progenitors, but not LMX1a. (N) TH+ MbDA neurons were depleted; inset shows sparse TH+ neurons. (O) Anterior 5-HT+ neurons were depleted whereas those at the edge of the En1-lineage domain (2) were diminished and those outside the En1-lineage domain (3) were unaffected. (P) Representative En1<sup>cre/+;Wnt1<sup>cre/+;R26tdTomato</sup> embryo with En1-lineage derived domain that was microdissected, sorted, and genotyped by PCR. En1-derived cells contained only the Wnt1<sup>fl/fl</sup> allele, cp, choroid plexus; dl, diencephalon; fl, forelimb; hi, hind limb; m, mammillary body; pros, prosencephalon; pt, pretectum; rp, Rathke’s pouch.

Fig. 3. Early deletion of Wnt1 depletes MbDA neurons. (A-H) En1<sup>fl/fl</sup>;Wnt1<sup>cre/+;R26tdTomato</sup> embryo at E12.5. (A,B) En1 lineage (red) contribution to the mes, r1, trigeminal ganglia (tg, asterisk) and craniofacial domain (cf). Axons traversed the tg and lateral a.Hb at the pontine flexure (yellow arrow). (C) Region of analysis (black box), v.Mes flexure (diamond) and En1-lineage (red shading). (D-H) Control sections immunolabeled for indicated markers (green) and the lineage tracer (dsred+, red). En1-derived cells were throughout the mes/r1 (delineated by arrowheads). The mes/r1 boundary (arrows) coincided with posterior limit of OTX2. (E) LMX1a in the ventricular zone (vz, yellow bracket) up to the anterior limit of the v.Mes (white arrowheads). (F) MbDA neurons in the differentiated domain (ds2, yellow bracket); inset shows TH+ neurons at higher magnification. (G,H) 5-HT+ neurons in v.r1. Numbers indicate rostral to caudal 5-HT+ neurons as indicated in text. (I-P) En1<sup>fl/fl</sup>;Wnt1<sup>cre/+;R26tdTomato</sup> mutants at E12.5 (IJ). Wnt1 mutants were devoid of the mes (indicated by red arrow), but did have a small fused mes/r1 domain (mes/r1*) and sparse projections at the pontine flexure (yellow arrow). (K) Region of analysis and En1-lineage (as described for C). (L-P) The En1-derived mes/r1 domain (red) was diminished, but a cohort of En1 lineage-derived cells was present. The anterior boundary was irregular and dsRed<sup>+</sup> cells were in the dl. (LM) Wnt1-mutant/En1-lineage derived domain had OTX2 progenitors, but not LMX1a. (N) TH+ MbDA neurons were depleted; inset shows sparse TH+ neurons. (O) Anterior 5-HT+ neurons were depleted whereas those at the edge of the En1-lineage domain (2) were diminished and those outside the En1-lineage domain (3) were unaffected. (P) Representative En1<sup>cre/+;Wnt1<sup>cre/+;R26tdTomato</sup> embryo with En1-lineage derived domain that was microdissected, sorted, and genotyped by PCR. En1-derived cells contained only the Wnt1<sup>fl/fl</sup> allele, cp, choroid plexus; dl, diencephalon; fl, forelimb; hi, hind limb; m, mammillary body; pros, prosencephalon; pt, pretectum; rp, Rathke’s pouch.

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way of the pontine flexure (supplementary material Fig. S6A,B, inset) consistent with previous studies (Ellisor et al., 2009). En1Cre;Wnt1ΔMHB/ΔMHB;R26TdT embryos were similar to controls and had trigeminal ganglia projections that innervated the craniofacial region and the pontine flexure (supplementary material Fig. S6C,D). However, analysis of whisker fields (location of vibrissae) and serotonergic innervation of the craniofacial region of conditional knockout embryos showed aberrantly patterned whisker fields innervated by serotonergic neurons and a severe truncation of the En1-derived lateral a.Hb (supplementary material Fig. S6C,D,G,H).

**Wnt1 in r1 is not required for Cb development and is transiently required for patterning**

We previously showed that late temporal epochs of Wnt1-expressing progenitors in the rhombic lip of r1 contribute to the Cb, consistent with late Wnt1 expression in this domain (Hagan and Zervas, 2012). Thus, r1 depletion in En1Cre;Wnt1ΔMHB/ΔMHB embryos could be due to Wnt1 function in the mes at the mes/r1 boundary or in the rhombic lip. We addressed this issue by deleting Wnt1 in the rhombic lip using Gbx2CreER-ires-eGFP mice (Chen et al., 2009) plus our Wnt1fl allele and the R26tdTomato line to simultaneously mutate and track the recombination event (Fig. 4A-L). Pregnant dams were treated with tamoxifen at E8.5 to mediate recombination in r1; Wnt1 was effectively deleted based on molecular analysis to detect the deleted allele and in situ hybridization to show loss of Wnt1 in r1 (Fig. 4A,B,G,H). We evaluated Gbx2 lineage contribution (dsred+) Gbx2(GFP), OTX2 and LMX1a expression in r1, which were unaffected by the loss of Wnt1 (Fig. 4C-F,I-L). Thus, the deletion of r1 was due to Wnt1 function in the mes and not in the rhombic lip. We next tested the hypothesis that Wnt1 continues to be required for patterning the mes/r1 and for MbDA neuron development using Wnt1-CreER mice (Zervas et al., 2004) coupled with tamoxifen to mediate recombination in Wnt1-expressing

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**Fig. 4. Temporal requirement of Wnt1 for patterning the Mb and Cb.** (A-F) Control E12.5 Gbx2CreER-ires-eGFP;Wnt1fl/+;R26tdTomato embryos received tamoxifen at E8.5 and were analyzed for the Gbx2 lineage, Wnt1, Gbx2(GFP), OTX2 and LMX1a. Arrowheads indicate limit of marker expression. (G-L) E12.5 Gbx2CreER-ires-eGFP;Wnt1fl/+;R26tdTomato mutants that received tamoxifen at E8.5 had a loss of Wnt1 in the upper rhombic lip (URL). The Gbx2 lineage, Gbx2(GFP), OTX2 and LMX1a were unaffected. Arrowheads indicate limit of marker expression. (M-X) Wnt1-CreER;Wnt1fl/+;R26tdTomato controls that received tamoxifen at E8.5 and analyzed at E12.5 showed the Wnt1 lineage (red) in the mes, trigeminal ganglia (asterisk) and craniofacial domain (cf). (O-R) Controls labeled for markers (green) and the lineage tracer (dsred+, red) showed Wnt1-derived cells throughout the v.Mes, but not in r1. The Wnt1 lineage abutted 5-HT+ neurons in vr1 and expressed OTX2 and LMX1a in the vz. Wnt1-lineage derived TH+ neurons in the dz. (S-X) Wnt1-CreER;Wnt1fl/+;R26tdTomato mutants had a smaller v.Mes versus controls although the Wnt1 lineage (red) was unaffected. 5-HT+ neurons were unaffected. Wnt1-derived cells expressed OTX2 and LMX1a, but caudal MbDA neurons were depleted (asterisk, bracket in X). cp, choroid plexus; Ctx, cerebral cortex; di, diencephalon; dz, differentiated zone; fl, forelimb; hl, hindlimb; m, mammillary body; pros, prosencephalon; vz, ventricular zone.
progenitors at E8.5 (Fig. 4M-X). Wnt1-derived cells (red fluorescence) were similarly distributed in the mes, posterior Hb, and spinal cord in both Wnt1-CreER;Wnt1\(^{\Delta MHB/+}\);R26\(^{\text{tdTomato}}\) and Wnt1-CreER;Wnt1\(^{\Delta MHB/+};\Delta vMes\);R26\(^{\text{tdTomato}}\) littermates at E12.5 (Fig. 4M,N,S,T). In control embryos, Wnt1-derived cells in the v.Mes abutted the 5-HT\(^{+}\) neurons in the a.Hb and co-expressed OTX2 and LMX1a (Fig. 4O-Q). TH\(^{+}\) MbDA neurons in Wnt1-CreER;Wnt1\(^{\Delta MHB/+};\Delta vMes\);R26\(^{\text{tdTomato}}\) embryos were located in the differentiating zone of the v.Mes with a posterior limit at the mes/r1 boundary (Fig. 4R). The v.Mes in Wnt1-CreER;Wnt1\(^{\Delta MHB/+};\Delta vMes\);R26\(^{\text{tdTomato}}\) embryos was smaller than that in controls and contained an abnormal notch, but was otherwise patterned normally based on the morphology of the v.Mes (Fig. 4U-X). The mutant Wnt1 lineage marked at E8.5 expressed OTX2 and LMX1a and was distributed similar to controls (Fig. 4V,W). However, the deletion of Wnt1 at E8.5 resulted in depletion of TH\(^{+}\) neurons in the medial v.Mes, but not of 5-HT\(^{+}\) neurons in v.r1 (Fig. 4U,X). Interestingly, we also observed an increase in TH\(^{+}\) neurons in the off-midline plane of Wnt1 conditional knockout mutants (data not shown). These phenotypes were observed in two out of four embryos with the variability probably arising from the mosaic nature of recombination with this transgenic line. We also administered tamoxifen to Wnt1-CreER;Wnt1\(^{\Delta MHB/+};\Delta vMes\);R26\(^{\text{tdTomato}}\) embryos at E10.5, which did not result in any overt phenotype in control and mutants at E12.5 (Fig. 4M,N,S,T). In addition, a subset of MbDA progenitors continued to require Wnt1 for their development between E8.5 and E10.5.

Wnt1 has a continued role in MbDA neuron development and regulates cell cycle exit

We used the Shh\(^{Cre}\) line (Harfe et al., 2004; Hayes et al., 2011; Tang et al., 2010; Tang et al., 2009) to address the continued role of Wnt1 because Shh\(^{Cre}\) mediates robust and cumulative recombination in the v.Mes beginning at E9.0-9.5 (Harfe et al., 2004; Hayes et al., 2011), which is later than with our En1\(^{Cre}\) mice. To validate that Shh\(^{Cre}\) was appropriate to delete Wnt1 we used FACS to sort v.Mes cells from Wnt1-Venus mice (Brown et al., 2011; Ellisor et al., 2012; Hagan and Zervas, 2012) and performed qRT-PCR that showed that Wnt1(GFP)-expressing MbDA neuron progenitors also express Shh (supplementary material Fig. S8). Because Shh\(^{Cre}\) mediates recombination in the v.Mes, we operationally define Wnt1\(^{vMes}\) as the recombined allele for this experiment (Fig. 5). The Shh lineage in E12.5 Shh\(^{Cre}\);Wnt1\(^{vMes/+};\Delta vMes\);R26\(^{\text{tdTomato}}\) embryos analyzed by whole-mount fluorescence and on immunolabeled sagittal sections had a typical distribution of OTX2\(^{+}\), LMX1a\(^{+}\) and NURR1\(^{+}\) progenitors and early differentiating TH\(^{+}\) neurons (Fig. 5A-G). Shh\(^{Cre}\);Wnt1\(^{vMes/+};\Delta vMes\);R26\(^{\text{tdTomato}}\) littermates had a normal distribution of the Shh lineage and OTX2\(^{+}\), LMX1a\(^{+}\) and NURR1\(^{+}\) progenitors (Fig. 5H-L). However, we observed a depletion of medial MbDA neurons and an expansion of more laterally positioned MbDA neurons in mutants (Fig. 5F,G,M,N). Quantification of medial populations revealed TH\(^{+}\) counts of 462±103 (mean±s.d.) versus 147±27 from controls and conditional mutants, respectively (n=4 each genotype), which was a significant decrease in MbDA neurons (Fig. 5O; P<0.0006). By contrast, off-midline TH\(^{+}\) counts were 192±51 and 288±45 from control and mutants, respectively, which was a corresponding significant

Fig. 5. Late temporal requirement of Wnt1 for MbDA neuron development. (A-G) Shh\(^{Cre}\);Wnt1\(^{vMes/+};\Delta vMes\);R26\(^{\text{tdTomato}}\) controls at E12.5 showed the Shh lineage (red) in the v.Mes. (C-G) Sections immunolabeled for indicated markers (green) and the lineage tracer (dsred\(^{+}\), red) showed that the Shh lineage expressed OTX2\(^{+}\), LMX1a\(^{+}\) and NURR1\(^{+}\) (E,G) Shh lineage-derived medial and off-midline TH\(^{+}\) neurons. Orange arrowheads indicate TH\(^{+}\) neurons. White arrows indicate MHB. Box in A' indicates the region shown in C-G. (H-N) Shh\(^{Cre}\);Wnt1\(^{vMes/+};\Delta vMes\);R26\(^{\text{tdTomato}}\) mutants did not have morphological abnormalities. (J-L) The v.Mes morphology was unaffected and Shh-derived cells expressed OTX2\(^{+}\), LMX1a\(^{+}\) and NURR1\(^{+}\), similar to controls. (MN) Medial TH\(^{+}\) MbDA neurons (arrowheads) were severely reduced whereas off-midline TH\(^{+}\) neurons were expanded in mutants. White arrows indicate the MHB. (O) Quantification of TH\(^{+}\) MbDA neurons in medial and off-midline planes including rostral to caudal positions from the v.Mes of control and conditional mutants (*P<0.05, **P<0.01, ***P<0.001). Error bars represent s.d. Asterisk in A' indicates trigeminal ganglia; cf, craniofacial; di, diencephalon; f, forelimb; h, hindlimb; m, mammillary body; pros, prosencephalon.
increase of off-midline MbDA neurons (Fig. 5O; *P*=0.020). These changes were observed at rostral, intermediate and caudal positions in the v.Mes (Fig. 5O). 

MbDA neurons differentiate such that early versus late born neurons occupy more lateral or medial positions, respectively (Kawano et al., 1995; Bayer et al., 1995; Bye et al., 2012). To test whether the MbDA neuron phenotype in Shh<sup>Cre</sup>; Wnt1<sup>fl/fl</sup> / En1<sup>Cre</sup>; Shh<sup>Cre</sup>; Wnt1<sup>fl/fl</sup> / R26<sup>TdT</sup>/Tomato embryos was caused by alterations in cell cycle exit we administered EdU at E11.5 and analyzed embryos 24 hours later by confocal microscopy and quantitative analysis to detect Hoechst (nuclear counter stain), dsred (Shh lineage), TH (terminally differentiated MbDA neurons), EdU (birthdating) and Ki67 (proliferation) (Fig. 6; supplementary material Fig. S9). Shh<sup>Cre</sup>; Wnt1<sup>fl/fl</sup> / En1<sup>Cre</sup>; Shh<sup>Cre</sup>; Wnt1<sup>fl/fl</sup> / R26<sup>TdT</sup>/Tomato controls had 160±33 (mean±s.d.) Shh lineage-derived medial MbDA neurons that differentiated at E11.5 (TH+/EdU+/Ki67<sup>+</sup>) (Fig. 6A-F,Y). By contrast, Shh<sup>Cre</sup>; Wnt1<sup>fl/fl</sup> / En1<sup>Cre</sup>; Shh<sup>Cre</sup>; Wnt1<sup>fl/fl</sup> / R26<sup>TdT</sup>/Tomato mutants had 38±14 medial MbDA neurons that differentiated at E11.5 (TH+/EdU+/Ki67<sup>+</sup>), which was a significant fourfold reduction (Fig. 6G-L,Y; *P*=0.0002). Control embryos had negligible 3±3 off-midline TH+/EdU+/Ki67<sup>+</sup> neurons that differentiated at E11.5 (Fig. 6M-R,Y) whereas mutant embryos had 38±26, which was a significant increase of MbDA neurons that had differentiated at E11.5 (Fig. 6S-X,Y; *P*=0.0200). Both control and mutant embryos had similar counts of proliferating progenitors (Ki67<sup>+</sup>). However, mutants had significantly more Ki67<sup>+</sup> progenitors compared with controls (420±152 versus 155±139, respectively; *P*=0.005). By contrast, the ratio of off-midline to midline TH+/EdU+/Ki67<sup>+</sup> neurons was 0.58±0.03 (Fig. 6Z, dark gray bars) whereas Shh<sup>Cre</sup>; Wnt1<sup>fl/fl</sup> / En1<sup>Cre</sup>; Shh<sup>Cre</sup>; Wnt1<sup>fl/fl</sup> / R26<sup>TdT</sup>/Tomato embryos had a ratio of 0.05±0.02 (Fig. 6Z, light gray bars), which was a significant reduction (P<0.005). By contrast, the ratio off-midline was 0.01±0.01 for controls (Fig. 6Z<sup>+</sup>, dark gray bars) and 0.08±0.07 for mutants (Fig. 6Z<sup>+</sup>, light gray bars), which was a significant increase (P<0.01). The rostral-to-caudal distribution showed similar results (Fig. 6Z<sup>+</sup>). Thus, the conditional deletion of Wnt1 resulted in a loss of medial MbDA neurons as well as a smaller fraction of medial cells that differentiated into MbDA neurons and a concomitant larger fraction of ectopic laterally positioned MbDA neurons (Fig. 6AA).

**DISCUSSION**

Wnt1 is important for Mb and a.Hb development (McMahon and Bradley, 1990; McMahon et al., 1992), but the role of Wnt1 at specific stages of embryonic development or within specific genetic lineages has been elusive because Wnt1 mutant mice that were previously available for analysis were derived by neomycin disruption (null alleles) or were caused by a spontaneous mutation (McMahon and Bradley, 1990; McMahon et al., 1992; Thomas and Capecci, 1990; Thomas et al., 1991). We circumvented these limitations by generating a Wnt1<sup>fl</sup> allele, which we used in spatial and temporal deletion experiments in combination with genetic lineage tracing. We show that Wnt1 patterns the d.Mes and v.Mes, and patterns r1 during an early and brief window (~12 hours between 0-1 somites and 6-8 somites). By tracing the En1 lineage in Wnt1 conditional mutants, we show that a large portion of En1-derived tissue is depleted at E12.5. However, cells derived from the En1 lineage are present in mutants with a more substantial allotment in v.Mes and v.r1 compared with dorsal tissue. The presence of an En1-derived Wnt1-mutant domain indicates that a cohort of cells derived from the En1 lineage does not require Wnt1. Thus, the Mb is derived from a heterogeneous pool of progenitors and is partitioned by complex molecular identities and genetic lineages. The deletion of Wnt1 in the rhombic lip of r1 did not phenocopy the truncation of r1, which provides definitive experimental evidence that Wnt1 in the mes is functionally required for r1 development, probably by regulating the organizer molecule FGF8.

OTX2<sup>+</sup> progenitors persist in En1-derived Wnt1-mutant v.Mes tissue, suggesting that signaling by Wnt1 does not induce or maintain Otx2 at least in a subset of v.Mes cells. This is in contrast to viral-mediated transduction of Wnt1 in ES cells, which increases Otx2 mRNA levels (Chung et al., 2009). Thus, Wnt1 regulation of Otx2 might be context dependent. The remaining En1-derived Wnt1-mutant domain is devoid of LMX1a<sup>+</sup> cells, suggesting that Wnt1-dependent progenitors (which may also be the LMX1a-expressing population) are unable to expand in the absence of Wnt1 or that a cohort of mutant progenitors may not have been induced resulting in a smaller initial progenitor pool. At the same time, a Wnt1-independent population may expand with many of those cells still expressing OTX2. We show that Wnt1 requirement for LMX1a progenitors is transient and occurs between the 1-somite and the 6- to 8-somite stages. This finding complements previous observations that Wnt1(GFP) is expressed more broadly and prior to LMX1a in the v.Mes (Brown et al., 2011). It has been proposed that Lmx1a functions as a determinant of MbDA neurons (Andersson et al., 2006); interestingly, WNT1 uses the canonical β-catenin pathway to regulate Lmx1a expression and, conversely, LMX1a binds directly to the promoter of Wnt1 during MbDA neuron differentiation (Chung et al., 2009). Thus, an alternative explanation for the phenotype of the early deletion of Wnt1 is that Wnt1 initiates Lmx1a expression followed by a short pulse of WNT1 and Lmx1a-Wnt1 cross-regulatory reinforcement. Later, an uncoupling of Lmx1a and Wnt1 might occur in MbDA neuron progenitors, as suggested by results of our conditional deletion of Wnt1 with Wnt1-CreER or Shh<sup>Cre</sup> lines. This dynamic regulation is consistent with how Wnt1 temporally regulates oscillations of gene expression and genetic pathway interactions in vitro (Wexler et al., 2011). We also show that even though a v.Mes domain persists with early Wnt1 deletion, TH<sup>+</sup> neurons are not generated, which is consistent with the absence of LMX1a. Interestingly, medial MbDA neurons have a continued requirement of Wnt1 with Wnt1-CreER or Shh<sup>Cre</sup> lines. This dynamic regulation is consistent with how Wnt1 temporally regulates oscillations of gene expression and genetic pathway interactions in vitro (Wexler et al., 2011). However, cells derived from the En1 lineage are present in mutants with a more substantial allotment in v.Mes and v.r1 compared with dorsal tissue. The presence of an En1-derived Wnt1-mutant domain indicates that a cohort of cells derived from the En1 lineage does not require Wnt1. Thus, the Mb is derived from a heterogeneous pool of progenitors and is partitioned by complex molecular identities and genetic lineages. The deletion of Wnt1 in the rhombic lip of r1 did not phenocopy the truncation of r1, which provides definitive experimental evidence that Wnt1 in the mes is functionally required for r1 development, probably by regulating the organizer molecule FGF8.

SHH and FGF8 function cooperatively to induce MbDA neurons during a short developmental window and together enhance the efficiency of producing MBDA-like neurons from ES cells and induced pluripotent stem cells (iPSCs) (Ye et al., 1998; Lee et al., 2000; Soldner et al., 2009). Wnt1-expressing v.Mes progenitors express components consistent with these signaling pathways and also express Fzd9 (supplementary material Fig. S8). Thus, we show that MbDA neuron progenitors are poised to respond to SHH, FGF8 and WNT1 signaling. Previous studies have shown that canonical
WNT signaling can antagonize Shh expression (Joksimovic et al., 2009). However, our early deletion of Wnt1 shows that Shh expression is not increased and suggests that WNT1 does not drive canonical WNT-mediated antagonism of Shh in the v.Mes and that the phenotype we observe is not due to changes in Shh. It is possible that the early deletion of Wnt1 and the near-complete loss of MbDA

![Image of cell cycle exit data](image.png)

**Fig. 6. Late conditional Wnt1 deletion alters cell cycle exit of MbDA neurons in vivo.** (A-X) EdU was administered at E11.5 and embryos were analyzed at E12.5. (A-F,M-R) ShhCre;Wnt1ΔvMes/+;R26Tomato controls. (G-L,S-X) ShhCre;Wnt1ΔvMes/+;R26Tomato mutants. Medial sections (A-L) and off-midline sections (M-X) are shown. Sections were co-labeled for Hoechst (A,G,M,S), dsred (B,H,N,T), TH (C,I,O,U), EdU (D,J,P,V) and Ki67 (E,K,Q,W). Overlay is shown in F,L,R.X. Medial domains of ShhCre;Wnt1ΔvMes/+;R26Tomato mutants had more cells in the active cell cycle at E11.5 (Ki67+, white arrows) compared with controls. Fewer cells exited the cell cycle and differentiated into MbDA neurons (EdU+/TH+/Ki67–, white arrowheads) in mutants versus controls. Yellow arrowheads indicate EdU+/TH+/Ki67– cells. Off-midline domains of ShhCre;Wnt1ΔvMes/+;R26Tomato mutants showed increased MbDA neurons birthdated at E11.5 (EdU+/TH+/Ki67–, yellow arrows). MbDA neurons born outside the range of EdU kinetics were EdU+/TH+/Ki67– (yellow arrows). Dashed line indicates boundary between ventricular zone (VZ) and differentiated zone (DZ). (Y,Z) Quantitative assessment of ShhCre;Wnt1ΔvMes/+;R26Tomato controls (dark gray bars) and ShhCre;Wnt1ΔvMes/ΔvMes;R26Tomato mutants (light gray bars) at E12.5. (Y,Z) Total, rostral, intermediate and caudal MbDA neurons birthdated at E11.5 (TH+/EdU+) in medial and off-midline planes. (Z,Z) Ratio of MbDA neurons born at E11.5 (TH+/EdU+/Ki67–) to the total number of cells born at E11.5 (EdU+/Ki67–). *P<0.05, **P<0.01, ***P<0.001. Error bars represent s.d. (AA) Illustration of cell cycle exit data. At E11.5, Ki67+ cells (green cells) proliferate in the VZ, subsequently migrate along radial glia (beige cells), and differentiate into TH+ MbDA neurons (gold neurons); the early born MbDA neurons are located more lateral than the later born MbDA neurons. The addition of EdU at E11.5 tracks differentiating MbDA neurons (red shading, gold neurons). In ShhCre;Wnt1ΔvMes/+ embryos, TH+/EdU+/Ki67– MbDA neurons were primarily located in a medial location whereas TH+/EdU+/Ki67– MbDA neurons were located laterally. The conditional deletion of Wnt1 in ShhCre;Wnt1ΔvMes/+ embryos disrupted the timing of cell cycle exit, which caused the depletion of medial MbDA neurons and a concomitant increase of ectopic, laterally positioned TH+/EdU+/Ki67– MbDA neurons.
neurons were secondary to the changes in Fgf8. However, we do not believe that this is the case because, unlike our En1Cre;Wnt1fl/fl conditional mutants, which have a near-complete depletion of MbDA neurons at E12.5, the deletion of Fgf8 (at the same time and using the same Cre line) in En1Cre;Fgf8fl/fl embryos results in a normal complement of TH+ neurons at E12.5 (Ellisor et al., 2012). Interestingly, ES cells treated with SHH and FGF8 express Wnt1 prior to becoming MbDA-like neurons (Lee et al., 2000) (M.Z., unpublished results) and Wnt1 is important for SHH- and FGF8-mediated ectopic induction of MbDA neurons (Prakash and Wurst, 2006). Therefore, the role of Wnt1 in MbDA neuron development is independent of Fgf8 and Shh, although future experiments are needed to address how the convergence of FGF8, SHH and WNT1 signaling shapes MbDA neuron progenitors.

Deletion of Wnt1 in the Shh lineage (ShhCre;Wnt1fl/fl) reveals that OTX2, LMX1a and NURR1 are unaffected, which is similar to observations in ShhCre;β-cateninfl/fl mice described by Tang et al. (Tang et al., 2009) using the same ShhCre line. We show that the cell cycle of only MbDA neurons are affected in our conditional Wnt1 mutants as reflected in the decreased ratio of MbDA neurons/all cells born. By contrast, conditional β-catenin mutants have a decrease in the birthdating of all cells, including MbDA neurons. These findings suggest that disrupting β-catenin-dependent WNT signaling causes a more global disruption of cells going through S phase than does the deletion of WNT1 ligand alone. The reduction of MbDA neurons born at E11.5 is similar in conditional β-catenin and our conditional Wnt1 mutants (~50% decrease in E11.5-born MbDA neurons in both mutants), suggesting that cell cycle progression and neurogenesis of MbDA neurons is largely mediated by WNT1 via canonical signaling. Interestingly, disruption of the cell cycle is linked to the depletion of differentiated MbDA neurons (as shown by decreased total numbers of TH+ neurons), and Wnt1 deletion in the v.Mes results in MbDA neurons that are displaced and improperly positioned, which may be due to changes in cell migration. Although this may shape MbDA neuron subtype identity, the early phenotype we observe does not directly address whether Wnt1 and cell cycle alteration affects ventral ventral segmental area (VTA) or substantia nigra (SNc) identity in adults, which will be important to resolve in future studies. In summary, our analyses reveal that Wnt1 is an important regulatory component that controls cell cycle exit and resolve the spatial and temporal roles for Wnt1 in specific developmental processes and in MbDA neurons during embryogenesis.

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References
and high-throughput Cre reporting and characterization system for the whole mouse brain. Nat. Neurosci. 13, 133-140.