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Genetic dissection of midbrain dopamine neuron development *in vivo*

Debra Ellisor [a], Caroline Rieser [a], Bettina Voelcker [a], Jason T. Machan [b], Mark Zervas [a,*,1]

[a] Department of Molecular Biology, Cell Biology and Biochemistry, Division of Biology and Medicine, Brown University, 70 Ship St., Providence, RI 02903, USA
[b] Departments of Orthopedics and Surgery at Rhode Island Hospital and the Warren Alpert Medical School at Brown University, Providence, RI 02903, USA

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ABSTRACT

Midbrain dopamine (MbDA) neurons are partitioned into medial and lateral cohorts that control complex functions. However, the genetic underpinnings of MbDA neuron heterogeneity are unclear. While it is known that *Wnt1*-expressing progenitors contribute to MbDA neurons, the role of *Wnt1* in MbDA neuron development *in vivo* is unresolved. We show that mice with a spontaneous point mutation in *Wnt1* have a unique phenotype characterized by the loss of medial MbDA neurons concomitant with a severe depletion of *Wnt1*-expressing progenitors and diminished LMX1a-expressing progenitors. *Wnt1* mutant embryos also have alterations in a hierarchical gene regulatory loop suggesting multiple gene involvement in the *Wnt1* mutant MbDA neuron phenotype. To investigate this possibility, we conditionally deleted *Gbx2*, *Fgf8*, and *En1/2* after their early role in patterning and asked whether these genetic manipulations phenocopied the depletion of MbDA neurons in *Wnt1* mutants. The conditional deletion of *Gbx2* did not result in re-positioning or distribution of MbDA neurons. The temporal deletion of *Fgf8* did not result in the loss of either LMX1a-expressing progenitors nor the initial population of differentiated MbDA neurons, but did result in a complete loss of MbDA neurons at later stages. The temporal deletion and species specific manipulation of *En1/2* demonstrated a continued and species specific role of Engrailed genes in MbDA neuron development. Notably, our conditional deletion experiments revealed phenotypes dissimilar to *Wnt1* mutants indicating the unique role of *Wnt1* in MbDA neuron development. By placing *Wnt1*, *Fgf8*, and *En1/2* in the context of their temporal requirement for MbDA neuron development, we further deciphered the developmental program underpinning MbDA neuron progenitors.

Introduction

Midbrain dopamine (MbDA) neurons mediate a diverse array of complex behaviors including cognition and motor control. MbDA neurons are located in the ventral Mb (v. Mb) and are broadly delineated as a medial group (the ventral tegmental area, VTA) and two bi-lateral cohorts (the substantia nigra *pars compacta*, SNc). In addition to their anatomical differences, MbDA neurons are also molecularly, biochemically, and physiologically distinct (Mendez et al., 2005; Parent, 1996; Thompson et al., 2005; Grimm et al., 2004). Clinically, the distinction between VTA and SNc is important because the aberrant function of VTA MbDA neurons and the loss of SNc MbDA neurons are central features of schizophrenia and Parkinson's disease, respectively (Fallon et al., 2003; Parent, 1996). However, little is known about the developmental genetics underpinning the partitioning of MbDA neurons into medial and lateral MbDA neuron cohorts.

The Mb is derived from the mesencephalon (mes), which is an embryonic compartment that is patterned through the interactions of numerous transcription factors and cell signaling molecules, which include *Otx2*, *Gbx2*, *En1/2*, *Fgf8*, *Shh*, and *Wnt1* (reviewed in Zervas et al., 2005). Notably these genes also play varying roles in the induction, positioning, differentiation, and survival of v. mes progenitors (reviewed in Goridis and Rohrer, 2002; Prakash and Wurst, 2006; Zervas et al., 2005). We previously marked and tracked MbDA progenitors using Genetic Inducible Fate Mapping (GIFM), which uncovered that MbDA neurons are derived from the *Wnt1* lineage originating in the v. mes (Zervas et al., 2004; Brown et al., 2011). Gain-of-function studies *in vitro* suggest that *Wnt1* has a role in MbDA neuron induction and differentiation (Castelo-Branco et al., 2003; Prakash and Wurst, 2006). Recently, chromatin immunoprecipitation-qPCR reveals that *Wnt1*directly regulates a MbDA neuron determinant, *Lmx1a*, through β-catenin signaling (Chung et al., 2009). However, whether *Wnt1* is required for MbDA neuron development *in vivo* has been elusive because

* Correspondence to: Laboratory of Developmental Neurobiology, Genetics and Neurological Disease, Department of Molecular Biology, Cell Biology and Biochemistry, Division of Biology and Medicine, Box G-E436, Brown University, Providence, RI 02903, USA. Fax: +1 401 863 9653.
E-mail address: Mark_Zervas@brown.edu (M. Zervas).
URL: http://research.brown.edu/myresearch/Mark_Zervas (M. Zervas).
[1] Courier delivery: Laboratories for Molecular Medicine, 70 Ship Street, Rm. 436, Providence, RI 02903, USA.

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mice homozygous for a targeted allele of *Wnt1* die perinatally and have a patterning defect concomitant with a complete deletion of the Mb and cerebellum (Cb) (McMahon and Bradley, 1990; Thomas et al., 1991; McMahon et al., 1992).

We utilized mice that have a point mutation in *Wnt1* (*Wnt1*$^{SW/SW}$) and a large deletion of the Mb and Cb, but live to adulthood (Lane, 1967; Sidman, 1968; Thomas et al., 1991). The v. Mb in *Wnt1*$^{SW/SW}$ mice has not been extensively studied. Here, we use *Wnt1*$^{SW/SW}$ mice to investigate the *in vivo* requirement of *Wnt1* for the establishment of MbDA neurons during embryogenesis. The initial patterning of the mes is intact in mutant mice although *Wnt1*-expressing MbDA neuron progenitors are significantly depleted prior to differentiation, which correlates with the loss of VTA MbDA neurons in adults. Because *Wnt1* is involved in a genetic hierarchical loop that is compromised in *Wnt1*$^{SW/SW}$ mutants, we used conditional gene inactivation to assess how the deletion of genes affected in mutant embryos impacted MbDA neuron development. The conditional deletion of *Fgf8*, *Gbx2*, or *En1*/*En2* after their well-defined roles in patterning did not phenocopy the *Wnt1*$^{SW/SW}$ MbDA neuron abnormalities. Our findings show that genes required for patterning the mes have distinct temporal roles in MbDA neuron development *in vivo*. Finally, *Wnt1*$^{SW/SW}$ mice are poised to be a value research tool to study how the absence of VTA MbDA neurons affects brain function and behavior.

Materials and methods

Transgenic, reporter, and mutant mice

Wnt1$^{SW/SW}$ mice (stock ♯ 000243) were obtained from The Jackson Laboratory on a mixed C57Bl/6 background. We outbred the mice onto a Swiss Webster background to improve the general litter sizes and the *Wnt1*$^{SW/SW}$ phenotype was indistinguishable between both strains. Our analysis was performed on the latter background, which we have maintained for the last eight years. *En1*^{Cre} mice (Kimmel et al., 2000) were used to mediate the conditional knockout (cko) of the following floxed alleles: *Gbx2*^{lox} (Li et al., 2002), *En1*^{lox} (Sgaier et al., 2007) and *Fgf8*^{lox} (Chi et al., 2003). In addition, we used *Rosa26*^{CreERT2};*En1*$^{lox/-}$;*En2*$^{GFPlox/-}$ mice (Cheng et al., 2010) and *En1*^{Den} mice (Hanks et al., 1998; Sgaier et al., 2007). *Fgf8*^{lox} mice were generously provided by G. Martin and all other lines were generously provided by A. Joyner. Mice were maintained and sacrificed according to the protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Brown University (IACUC ♯0909081). *Wnt1-Venus* transgenic mice were generated by placing a *GFP* variant, *Venus* that encodes *YFP*, under the control of Wnt1 regulatory elements (See Brown et al., 2011 for details). This was done by replacing the *CreER*^{T} cassette with *YFP* in the vector used to generate *Wnt1-CreER*^{T} transgenic mice (Zervas et al., 2004). Embryos were staged in reference to embryonic day (E)0.5 being prior to noon of the day of the appearance of a vaginal plug.

Genotyping of Wnt1$^{+/+}$*;Wnt1-Venus, or Wnt1*$^{SW/+}$*;Wnt1-Venus, and Wnt1*$^{SW/SW}$*;Wnt1-Venus embryos for Fluorescent Activated Cell Sorting (FACS)*

A tail biopsy was obtained from each embryo prior to pooling to confirm the genotype of samples used for FACS. Because the *Wnt1-Venus* transgene has *YFP* placed between the untranslated region (UTR) and translated region of exon 1 and the transgene also contains the sequence for *Wnt1* (See Brown et al., 2011 for details) essentially all homozygotes appear as heterozygotes when genotyping using a routine PCR strategy. To overcome this issue we designed a three-phase PCR/restriction digest strategy to unambiguously identify *Wnt1*$^{SW/SW}$ embryos. First, we used the primers p*Wnt1.F3* (*ACAGCAACCACAGTCGTC*) and *Wnt1*ex2_3rev (*CTGAGATAGGGACATTCGG*), which anneal to the UTR in exon 1 and in the intronic region upstream of exon 3, respectively. These primers amplify a 2 kilobase (kb) amplicon corresponding to endogenous *Wnt1* alleles or a 3 kb amplicon corresponding to the *Wnt1-Venus* transgene (due to the YFP insert). The two amplicons were separated on a 1.4% agarose gel and the 2 kb band was gel extracted using the Qiaquick gel extraction kit. Subsequently, a second PCR reaction was done using the *Wnt1.sense* (*AGGAACCTCTTTGCCCTCAACC*) and *Wnt1.anti-sense* (*AAGTTCATCTGCACCACCG*) primers followed by BSLI restriction digest to discern between the *Wnt1*$^{+/+}$, *Wnt1*$^{SW/+}$, and *Wnt1*$^{SW/SW}$ alleles. The point mutation in the *Wnt1*^{SW} allele creates a distinct BSLI digest pattern, which always matched the embryos originally identified by phenotyping as described above.

Tissue preparation, in situ hybridization, and immunofluorescent immunocytochemistry (IF-ICC)

All embryos and adult tissues were prepared as previously described (Ellisor et al., 2009; Brown et al., 2011). Briefly, embryos were fixed in 4% paraformaldehyde (PFA) overnight (o/n) at 4 °C. Embryos were processed for whole mount *in situ* hybridization or were embedded in OCT as previously described (Ellisor et al., 2009). Cryoprotected tissue was frozen using 2-methyl-butane/acetone (http://home.primus.com.au/royellis/fr.htm) and blocks were stored at −20 °C. Embryonic material was cryosectioned at 10–12 μm and collected directly onto slides and stored at −20 °C until used. For adult tissue, mice were deeply anesthetized with beuthanasia-D. Once mice were unresponsive to deep hind paw pinch, the mice were flushed with saline and fixed with PFA by intracardiac perfusion as previously described (Brown et al., 2009). Brains were immersion fixed overnight (o/n) and stored in PBS. Adult sections (40 μm) were obtained with a Leica VT1000S vibratome and stored free floating at stored at 4 °C until used. Protocol details are available at the Zervas Lab web page (http://research.brown.edu/myresearch/Mark_Zervas). Whole mount RNA *in situ* hybridization was performed on E8.5, E9.5, and E10.5 embryos using antisense digoxigenin labeled RNA probes as previously described (Zervas et al., 2004; Ellisor et al., 2009). Sections for IF-ICC were fixed for five minutes, washed in PBT five times, and blocked in 10% donkey serum/PBT for two hours at room temperature. Sections were incubated with primary antibody (Ab) in 10% donkey serum o/n at 4 °C. The next day sections were washed in PBT five times. Subsequently, sections were incubated with secondary Abs in 1% donkey serum/PBT for two hours, washed in PBT five times, and counterstained with Hoechst nuclear dye (Molecular Probes) for one minute. Images were collected and processed using Open Lab, Magnafire, or Volocity 5.2 (Improvision) software and Adobe Photoshop or Illustrator CS2.

Primary antibodies: Dopaminergic, serotonergic, and cholinergic neurons were detected with anti-tyrosine hydroxylase (TH, Chemicon; 1:500), anti-5-hydroxytryptamine (5-HT, Jackson ImmunoResearch; 1:500), and anti-choline acetyl transferase (ChAT, Chemicon; 1:100), respectively. Calbindin (CALB) and g-protein inward rectifying potassium (K) channel 2 (GIRK2) were detected with anti-CALB (Swant; 1:1000) or anti-Girk2 (Alomone labs; 1:80), respectively. LMX1a Ab was generously provided by Michael German (UCSF, 1:1000). Secondary antibodies (Molecular Probes; all used at 1:500): donkey anti-mouse IgG-AMCA, donkey anti-mouse IgG-Alexa350, donkey anti-mouse IgG-Alexa555, donkey anti-mouse IgG-Alexa488, donkey anti-rabbit IgG-Alexa488, donkey anti-goat IgG-Alexa555.