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The widely used *Wnt1-Cre* transgene causes developmental phenotypes by ectopic activation of Wnt signaling

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Abstract

The *Wnt1-Cre* transgenic mouse line is extensively used in the study of the development of the neural crest and its derivatives and the midbrain. The *Wnt1* gene has important developmental roles in formation of the midbrain–hindbrain boundary, regulation of midbrain size, and neurogenesis of ventral midbrain dopaminergic (mDA) neurons. Here, we report that *Wnt1-Cre* transgenic mice exhibit phenotypes in multiple aspects of midbrain development. Significant expansion of the midbrain and increased proliferation in the developing inferior colliculus is associated with ectopic expression of *Wnt1*. Marked elevation of *Wnt1* expression in the ventral midbrain is correlated with disruption of the differentiation program of ventral mDA neurons. We find that these phenotypes can be attributed to ectopic expression of *Wnt1* from the *Wnt1-Cre* transgene leading to the ectopic activation of canonical Wnt/ β -catenin signaling. Since these caveats could complicate the utility of *Wnt1-Cre* in some developmental circumstances, we report a new *Wnt1-Cre2* transgenic mouse line that can serve the same purposes as the original without the associated phenotypic complications. These studies reveal an important caveat to a widely-used reagent, provide an improved version of this reagent, and indicate that the original *Wnt1-Cre* transgenic mouse line may be useful as a gain of function model for interrogating Wnt signaling mechanisms in multiple aspects of midbrain development.

Keywords

Neural crest; *Wnt1-Cre*; Midbrain; Wnt signaling; Dopaminergic neurons; Craniofacial

Introduction

The *Wnt1-Cre* mouse transgenic line is widely used for interrogating gene function and cell lineage relationships in a variety of developmental contexts including early neural crest migration, secondary palate, calvaria, cardiac outflow tract, and midbrain development (Brewer et al., 2004; Chai et al., 2000; Ito et al., 2003; Jiang et al., 2000, 2002; Katayama et

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ydbio.2013.04.026>.

al., 2011; Merrill et al., 2006; Tallquist and Soriano, 2003; Yoshida et al., 2008). Its utility is based on the fact that the *Wnt1* gene is highly expressed in the dorsal neural tube prior to the emigration of the neural crest and in the midbrain–hindbrain boundary (MHB) at the initiation of midbrain development. The *Wnt1-Cre* line, in combination with various *Cre* reporters, has thus been heavily employed for lineage tracing studies of neural crest cells and the midbrain.

Wnt1 has multiple roles in midbrain development. Inactivation of the *Wnt1* gene in mice resulted in loss of the entire mid/hindbrain region (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). Ectopic expression of *Wnt1* throughout the CNS under the control of the HoxB4 enhancer dramatically increased mitogenesis without altering patterning (Dickinson et al., 1994). Further, when *Wnt1* was ectopically expressed under the control of the endogenous *engrailed1* promoter in *En1^{Wnt1}* mice, pattern formation in the MHB was maintained, but increased proliferation and drastic overgrowth of the caudal-dorsal midbrain were observed (Panhuysen et al., 2004).

Ventral midbrain dopaminergic (mDA) neurons develop in close proximity to the MHB, a source of *Wnt1* expression, and ultimately modulate a broad range of neural processes, including movement, cognition and reward; their dysfunction leads to severe neurological disorders such as Parkinson's disease (PD). Therefore, understanding the molecular control of mDA differentiation is critical in the ultimate goal of therapeutic mDA neuron production, engraftment and function. The mDA differentiation program can be divided into three distinct steps (Prakash and Wurst, 2006b). First, induction of a progenitor field within the neuroepithelium that is competent to generate mDA precursors occurs between E8.5–E10.5 in the mouse and is characterized by the expression of general proneural genes such as the *Ngn2* transcription factor. Second, the specification of postmitotic mDA precursors marked by expression of markers such as the nuclear receptor family member *Nurr1* occurs at E10.5–E12.5. Finally, terminal differentiation begins by E12.5 and is characterized by the expression of tyrosine hydroxylase (TH), the rate-limiting enzyme in DA synthesis (Prakash and Wurst, 2006a, 2006b). Wnt signaling has been shown to have critical roles in the second and third steps of mDA neurogenesis, but has not been implicated in the first (Prakash et al., 2006; Tang et al., 2009). It is clear that *Wnt1* is involved in establishment of the committed mDA progenitor domain because ectopic *Wnt1* expression in the ventral hindbrain induced ectopic mDA neurons and because its expression was necessary for ectopic induction of mDA neurons by Fgf8 and Shh in ventral forebrain explants (Prakash et al., 2006). Loss of the obligate downstream Wnt signaling mediator β -catenin within the ventral midbrain in *Shh-Cre; β -catenin^{fl/fl}* embryos led to a dramatic reduction in mDA neurogenesis without changes in ventral midbrain patterning, whereas the later loss of β -catenin in differentiating mDA neurons revealed a role in the final step of mDA differentiation (Tang et al., 2009). Interestingly, hyperactivation of the pathway within the ventral midbrain in *Shh-Cre; β -catenin^{Ex3}* embryos resulted in a loss of mDA neurons, possibly by preventing cell cycle exit and differentiation of Ngn2-expressing committed progenitors to postmitotic progenitors (Tang et al., 2010). In these embryos, the induction of an mDA-competent progenitor field occurred and expression of *Ngn2* and *Nurr1* were slightly expanded. Taken together, these studies established the importance of Wnt signaling during midbrain development. During our studies of gene function in neural crest, we discovered that midbrain development is perturbed by the *Wnt1-Cre* transgene; since this transgene is so widely used, we investigated these phenotypes in greater depth.

Materials and methods

Genotyping

DNA was isolated from either tail or embryonic yolk sac samples, depending upon embryonic stage. qPCR was performed for the *Wnt1-Cre* transgene using primers in the second and third introns of *Wnt1* to determine quantity of transgene relative to the *wild-type* locus: 5'-CCA-CCT-CTT-CGG-CAA-GAT-CG-3' forward and 5'-GCT-AGA-AAG-AAT-CTG-GTG-CTG-ACC-3' reverse. This genotyping was conducted using *Bambi* as a reference gene for copy number.

Histology, immunofluorescence and in situ hybridization

Embryos for histology were fixed in Bouin's fixative, dehydrated through graded ethanol concentrations and embedded in paraffin. Sections were subsequently cut at 7 µm thickness and stained with hematoxylin and eosin. Embryos for immunofluorescence and *in situ* hybridization were fixed in 4% PFA, dehydrated to 25% sucrose and embedded in OCT; sections were cut at a thickness of 12 µm. Immunostaining was performed according to standard protocols using antibodies recognizing tyrosine hydroxylase (1:250; Millipore cat# AB152), BrdU (1:200; Abcam AB6326), Lef1 (1:75; Cell Signaling Technology product# 2230), and CyclinD1 (1:200; ThermoScientific cat# RB-010-P*). For BrdU experiments, pregnant dams were injected with 50 µg BrdU per gram body weight at 20 or 30 min prior to euthanasia for E12.5 or E15.5 embryos, respectively. *In situ* hybridization was performed according to standard protocols. *In situ* hybridized slides were counterstained with Orange G (Fig. 3; Supplementary Fig. 3; Supplementary Fig. 4I–K) or Nuclear Fast Red (Supplementary Fig. 1). The *Wnt1 in situ* probe hybridizes to base pairs 437–852 on the *Wnt1* mRNA (RefSeq NM_021279), corresponding to the entire second exon and roughly 60% of the third exon.

RT-PCR, qRT-PCR and western blots

Total RNA was extracted from snap frozen E12.5 and E13.5 embryos, according to Invitrogen's TRIzol Reagent protocol. cDNA was synthesized using Invitrogen's First-Strand Synthesis kit using random hexamers to prime the reverse transcriptase. *Wnt1-Cre* transcript sequencing was performed on RT-PCR products obtained using the following primer sets: *Wnt1* 5' utr 5'-CTC-ATT-GTC-TGT-GGC-CCT-GAC-C-3' forward with *Cre* 5'-ACG-CCT-GGC-GAT-CCC-TGA-AC-3' reverse, and *Cre* 5'-CGC-TGG-AGT-TTC-AAT-ACC-GG-3' forward with *Wnt1* exon 3 5'-CGG-AGG-TGA-TTG-CGA-AGA-TG-3' reverse. *Wnt1-Gal4* transcript sequencing was performed on RT-PCR products obtained according to the above scheme, replacing *Cre* primers with the following Gal4 primers: 5'-GTC-GGC-AAA-TAT-CGC-ATG-CTT-G-3' reverse and 5'-TTC-AAA-ACC-ACT-GTC-ACC-TGG-3' forward. Expression analysis of total *Wnt1* was carried out by qRT-PCR with *Wnt1* primers in the third and fourth exons (e3e4): 5'-CAT-CTT-CGC-AAT-CAC-CTC-CG-3' forward and 5'-GTG-GCA-TTT-GCA-CTC-TTG-G-3' reverse. Endogenous *Wnt1* expression was quantified using a *Wnt1* primer pair in the first and second exons (e1e2): 5'-ACA-GTC-GTC-AGA-ACC-GCA-GC-3' forward and 5'-TCA-ACA-GGT-TCG-TGG-AGG-AGG-C-3' reverse. These experiments were conducted using *GAPDH* as a reference gene. For protein quantification, E14.5 heads were homogenized in NP40 buffer and subjected to 10% SDS-PAGE and western blotting according to standard techniques. Blots were probed with *Wnt1* (Abcam cat# ab15251) and non-muscle myosin heavy chain IIA (Covance cat# PRB-440) antibodies and analyzed using an ImageQuant LAS 4000 scanner and software (GE).

Generation of *Wnt1-Cre2* transgenic mice

Wnt1 promoter and enhancer sequences were isolated from the pWEXP3C plasmid (Danielian and McMahon, 1996). The 1.3 kb *Wnt1* 5' promoter sequence was isolated by digestion with NotI and EcoRV from the pWEXP3C plasmid and placed upstream of a cDNA encoding *Cre* recombinase, followed by a 5.5 kb BglIII fragment that includes the *Wnt1* 3' enhancer. Transgenic lines were backcrossed to 129S4 mice and examined for *Cre* activity by crossing to ROSA26R reporter mice (Soriano, 1999). Allele specific genotyping of *Wnt1-Cre2* transgenic mice was performed by PCR with primers (5'-ACA-GCG-AAC-CAT-GCT-GCC-TG-3') and (5'-CAT-GTC-CAT-CAG-GTT-CTT-GC-3') giving a 465 bp product. The *Wnt1-Cre2* mice (129S4-Tg(*Wnt1-cre*)1Sor/J) are being deposited to the Jackson Laboratory Mouse Repository and will be available under Stock# 22137.

Results and discussion

Abnormal *Wnt1* expression and its phenotypic consequences in *Wnt1-Cre* mice

Over the course of our studies, we came to appreciate that *Wnt1-Cre^{Tg/+}* embryos display a marked enlargement of the midbrain beginning at E11.5, concomitant with dilation of the midbrain aqueduct (Fig. 1A–F). At E15.5, the inferior colliculi were thin and expanded, with increased CyclinD1 expression and overt cell proliferation as measured by BrdU incorporation, phenotypes that were exacerbated in *Wnt1-Cre^{Tg/Tg}* brains (Supplementary Fig. 1A–C,G–I). Moreover, at E12.5, *Wnt1-Cre^{Tg/+}* embryos also displayed a reduction in TH-expressing mDA neurons, which were nearly completely absent from *Wnt1-Cre^{Tg/Tg}* embryos (Supplementary Fig. 2A–C). These phenotypes were completely penetrant and consistent in *Wnt1-Cre* mice obtained directly from Dr. A.P. McMahon or from the Jackson Laboratories (Tg(*Wnt1-cre*)11Rth Tg(*Wnt1-GAL4*)11Rth/J), on 129S4 and C57Bl/6J backgrounds.

To understand the basis for these phenotypes, we examined *Wnt1* expression by *in-situ* hybridization and found that at E12.5, *Wnt1* consistently exhibited elevated expression in the dorsal neural tube and ectopic expression in the caudolateral neuroectoderm of the mesencephalon (Fig. 1G–L). In addition, *Wnt1* exhibited expanded expression in more rostral sections of the ventral midbrain, adjacent to regions with altered TH staining (Fig. 3A–F; Supplementary Fig. 2D–F). Furthermore, the pattern of misexpression of *Wnt1* was identical to that of the *Cre* transcript in *Wnt1-Cre* embryos and correlated with the positions of midbrain expansion and mDA neuron loss (Fig. 1M–O; Supplementary Fig. 2A–C). These results indicate that *Wnt1-Cre* transgenic mice exhibit ectopic upregulation of *Wnt1* that correlates with the midbrain phenotypes that we observe.

Ectopic expression of *Wnt1* from the *Wnt1-Cre* transgene

The *Wnt1-Cre* transgene was generated by inserting a *Cre* recombinase cDNA between a 1.3 kb upstream element including the promoter and the *Wnt1* coding sequence, destroying the *Wnt1* initiation ATG in the process. This was followed by a 5.5 kb downstream genomic sequence that carries *Wnt1* enhancer elements (Danielian et al., 1998). This transgene was co-injected with a *Wnt1-Gal4* transgene constructed using a similar strategy (<http://jaxmice.jax.org/strain/003829.html>). To determine the basis for the misexpression of full length *Wnt1*, we performed a series of RT-PCR sequence experiments, identifying two transgene-specific species of *Wnt1* transcript in *Wnt1-Cre^{Tg}* embryos (Fig. 2A). The first of these included the 5' UTR of *Wnt1* followed by *Cre* and the 3' part of exon 1 spliced normally to the remaining 3' exons. Although the *Wnt1* ATG initiator codon was mutated to ATC, we found an in-frame ATG located in the vector sequence 165 bp upstream of the usual *Wnt1* start codon. This reading frame is predicted to encode a protein with 55 additional N-terminal amino acids, which would be cleaved as part of the signal sequence. A

second transgene-specific transcript instead incorporated the *Gal4* gene sequence into the first exon of *Wnt1* but did not possess an in-frame ATG that could explain *Wnt1* expression.

Using quantitative RT-PCR, we found that total *Wnt1* transcript was between 10.3 and 67.4 fold more abundant in E14.5 *Wnt1-Cre^{Tg/+}* embryonic heads and 84.5 fold more abundant in *Wnt1-Cre^{Tg/Tg}* than in controls (Fig. 2B). To test whether this transgene might also be affecting expression of the endogenous *Wnt1* locus, we performed quantitative RT-PCR to differentiate between endogenous *Wnt1* transcript and total transcript and found that endogenous *Wnt1* transcript remained similar to wild-type levels in *Wnt1-Cre^{Tg}* embryos. Western blot analysis of E14.5 embryonic head lysates confirmed an increase in Wnt1 protein correlating with genotype (Fig. 2C). Since Wnt1 is typically an activator of canonical Wnt/ β -catenin signaling, we examined the expression of the downstream transcriptional targets *Axin2* and *Lef1*; these two genes were also upregulated in the regions of ectopic Wnt1 upregulation (Fig. 1P–U; Supplementary Fig. 1D–I; Supplementary Fig. 2G–I). Taken together, these results indicate that ectopic Wnt1 expressed from the *Wnt1-Cre* transgene activates canonical Wnt/ β -catenin signaling, possibly leading to the midbrain phenotypes that we observe.

Ectopic Wnt signaling perturbs the first step of the mDA differentiation program

To begin to determine the developmental mechanisms by which elevated Wnt1 expression in the ventral midbrain (vMb) disrupts mDA neurogenesis, we examined expression of key markers in a developmental sequence from E11.5 to E13.5. At each of these stages, dramatically elevated Wnt1 expression was observed in the vMb of *Wnt1-Cre^{Tg/Tg}* embryos, correlating with loss of TH-expressing mDA neurons (Fig. 3A–L) and increased cell proliferation in the vMb of E12.5 *Wnt1-Cre^{Tg/Tg}* embryos (Supplementary Fig. 3D–F). Examination of the generic proneural marker *Ngn2* revealed a striking loss of expression in *Wnt1-Cre^{Tg/Tg}* at E11.5, specifically within the domain of Wnt1 overexpression (Fig. 3B,N). Whereas the domain of *Ngn2* expression was still perturbed within the domain of Wnt1 overexpression at E12.5, nearly complete recovery of expression was observed at E13.5, at which point a few TH-positive mDA neurons had also formed (Fig. 3O–R). The expression of *Nurr1*, a marker of postmitotic mDA progenitors and a gene necessary for the expression of TH in mature mDA neurons (Prakash andWurst, 2006b), was completely absent from the vMb at E11.5 and dramatically reduced in E12.5 *Wnt1-Cre^{Tg/Tg}* embryos compared with control (Fig. 3S–V). Again, recovery of *Nurr1* expression was observed by E13.5, when some TH-expressing neurons were apparent (Fig. 3W–X). Taken together, these results provide evidence that elevated Wnt/ β -catenin signaling in the vMb of *Wnt1-Cre^{Tg/Tg}* embryos causes extended maintenance of a proliferative, mDA-incompetent domain. This idea is consistent with findings that treatment of rat ventral midbrain precursor cultures with Wnt1-conditioned media increased the total number of neurons by affecting proliferation and not the proportion of TH-positive cells (Castelo-Branco et al., 2003). Our results suggest that Wnt/ β -catenin signaling levels must be tightly controlled to allow the correct initiation of an mDA-competent precursor domain in the vMb and indicate that the *Wnt1-Cre^{Tg/Tg}* mouse line may be a useful tool in the detailed study of this process.

Generation and characterization of a new Wnt1-Cre transgenic mouse

Because the *Wnt1-Cre* transgene has been so transformative for studies of neural crest and midbrain development, we adapted the original strategy for the generation of this transgene to generate a new *Wnt1-Cre2* transgenic mouse line (129S4-Tg(Wnt1-cre)1Sor/J) that would avoid the complications of ectopic *Wnt1* expression. This line includes the same 1.3 kb 5 promoter sequence, *Cre*, and a 5.5 kb 3' enhancer sequence without the *Wnt1* gene sequence itself. This transgene is similar to a transgenic line that has been previously shown to exhibit Wnt1-restricted *LacZ* reporter activity (Echelard et al., 1994). We screened founders by

PCR and verified insertion of the *Wnt1-Cre2* transgene in four of these lines by Southern blot (data not shown). Crossing these new *Wnt1-Cre2* founders with ROSA26R *Cre* reporter mice indicated that these transgenic lines recapitulate *Cre* recombinase activity in both the cardiac and cranial neural crest (Fig. 4; Supplementary Fig. 4C–F). More detailed characterization of one of these lines revealed similar recombination patterns to the original *Wnt1-Cre* in the branchial arches and cardiac outflow tract (Fig. 4B–I; Supplementary Fig. 4C–F), two tissues with well-characterized neural crest contributions. Although some recombination was also detected in branchial arch and frontonasal epithelium of *Wnt1-Cre2; ROSA26R^{+/-}* embryos, closer examination revealed similar epithelial activity of the original *Wnt1-Cre* transgenic line (Supplementary Fig. 4A–B). This pattern of staining has been found to be reproducible over four backcross generations to 129S4, indicating that expression of *Cre* is stable in this transgenic line. Importantly, the *Wnt1-Cre2* line does not exhibit the ectopic *Wnt1* expression or midbrain enlargement (Supplementary Fig. 4I–K) present in the original *Wnt1-Cre* transgenic mouse. Taken together, these observations indicate that the *Wnt1-Cre2* line will be a suitable replacement to achieve deletion in the neural crest cell lineages.

Conclusion

The generation of the *Wnt1-Cre* transgene was pioneering, and its broad application in midbrain and neural crest studies has transformed multiple fields of developmental biology. As such, it is critical to note the caveat of ectopic activation of Wnt/ β -catenin signaling that we describe here. These phenotypes are consistent with known roles for Wnt/ β -catenin signaling in these contexts and indicate that ectopic activation of Wnt/ β -catenin signaling may pose a confounding factor for some studies involving this mouse line (Dickinson et al., 1994; Panhuysen et al., 2004; Prakash et al., 2006; Tang et al., 2009, 2010). The *Wnt1-Cre* line may continue to prove useful in contexts that have been verified to lack ectopic activation of Wnt/ β -catenin signaling. This promoter construct has also been used to drive the expression of other genes during midbrain development (Danielian and McMahon, 1996; Lee et al., 1997); we have no information on whether those studies would be influenced by the findings presented here. Finally, the effect of misexpression of *Wnt1* on mDA neurons may make *Wnt1-Cre* transgenic mice a useful model for studying the differentiation of mDA neurons with the purpose of designing restorative therapies for Parkinson's disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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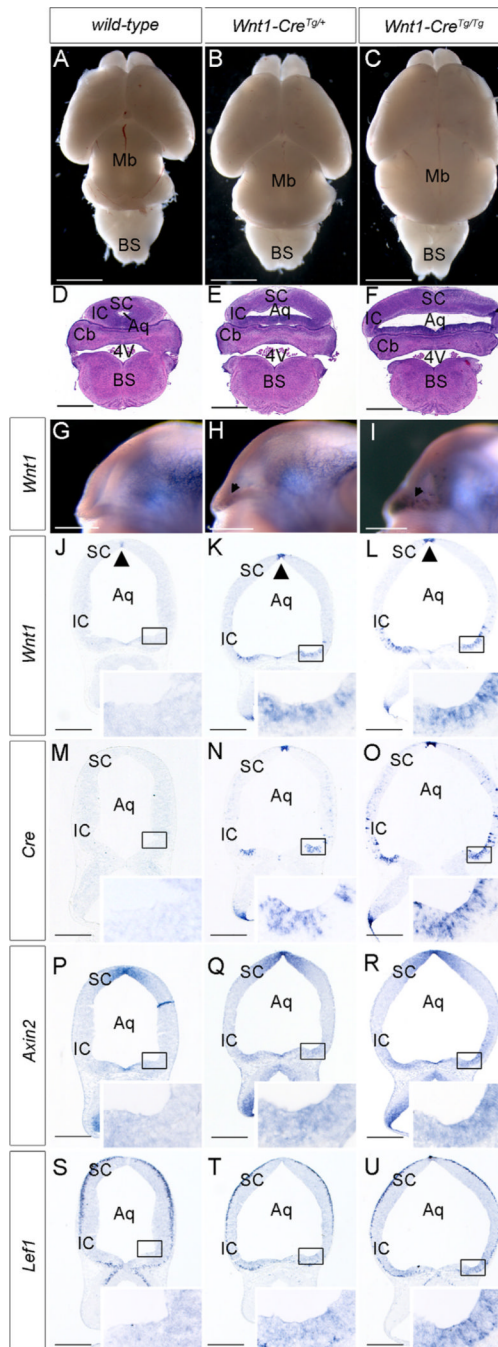


Fig. 1.

Wnt1 misexpression activates ectopic canonical Wnt/ β -catenin signaling and disrupts midbrain development in *Wnt1-Cre* transgenic mice. (A–C) Whole mount view of P0 brains reveals slightly expanded midbrain in *Wnt1-Cre^{Tg/+}* mice (B) and dramatically expanded midbrain in *Wnt1-Cre^{Tg/Tg}* mice (C) compared with *wild-type* (A). (D–F) Histological coronal sections of E18.5 mouse brains show dramatic expansion of the midbrain in *Wnt1-Cre^{Tg/+}* mice (E) and more severe expansion in *Wnt1-Cre^{Tg/Tg}* mice (F). This expansion mostly affects the inferior colliculus and is accompanied by its thinning and increased nuclear density (arrowhead in F). (G–I) Whole mount *in-situ* hybridization of E12.5 embryos reveals punctate misexpression of *Wnt1* around the caudolateral edge of the

midbrain–hindbrain boundary in *Wnt1-Cre^{Tg/+}* (arrows in H) and *Wnt1-Cre^{Tg/Tg}* embryos (arrows in I) compared with *wild-type* (G) (J–L). *In situ* hybridization of E12.5 coronal sections reveals ectopic *Wnt1* expression in the caudolateral aspect of the mesencephalon in *Wnt1-Cre^{Tg/+}* embryos (K), with increased intensity and extent in *Wnt1-Cre^{Tg/Tg}* embryos (L) (inset boxes in J–L). The expression of *Wnt1* in the dorsal aspect of the midbrain is also increased in intensity at these stages (arrowheads, J–L). (M–U) *In situ* hybridization of sections adjacent to J–L reveals *Cre* expression (M–O), *Axin2* upregulation (P–R), and *Lef1* upregulation (S–U) in the caudolateral aspect of the mesencephalon of *Wnt1-Cre^{Tg/+}* (N,Q,T) and *Wnt1-Cre^{Tg/Tg}* (O,R,U) embryos in regions corresponding to those with ectopic expression of *Wnt1*. Scale bars: (A–C) 2 mm, (D–F) 1 mm, (G–U) 500 μ m. (SC) superior colliculus, (IC) inferior colliculus, (Aq) aqueduct, (Cb) cerebellum, (4 V) fourth ventricle, (BS) brain stem.

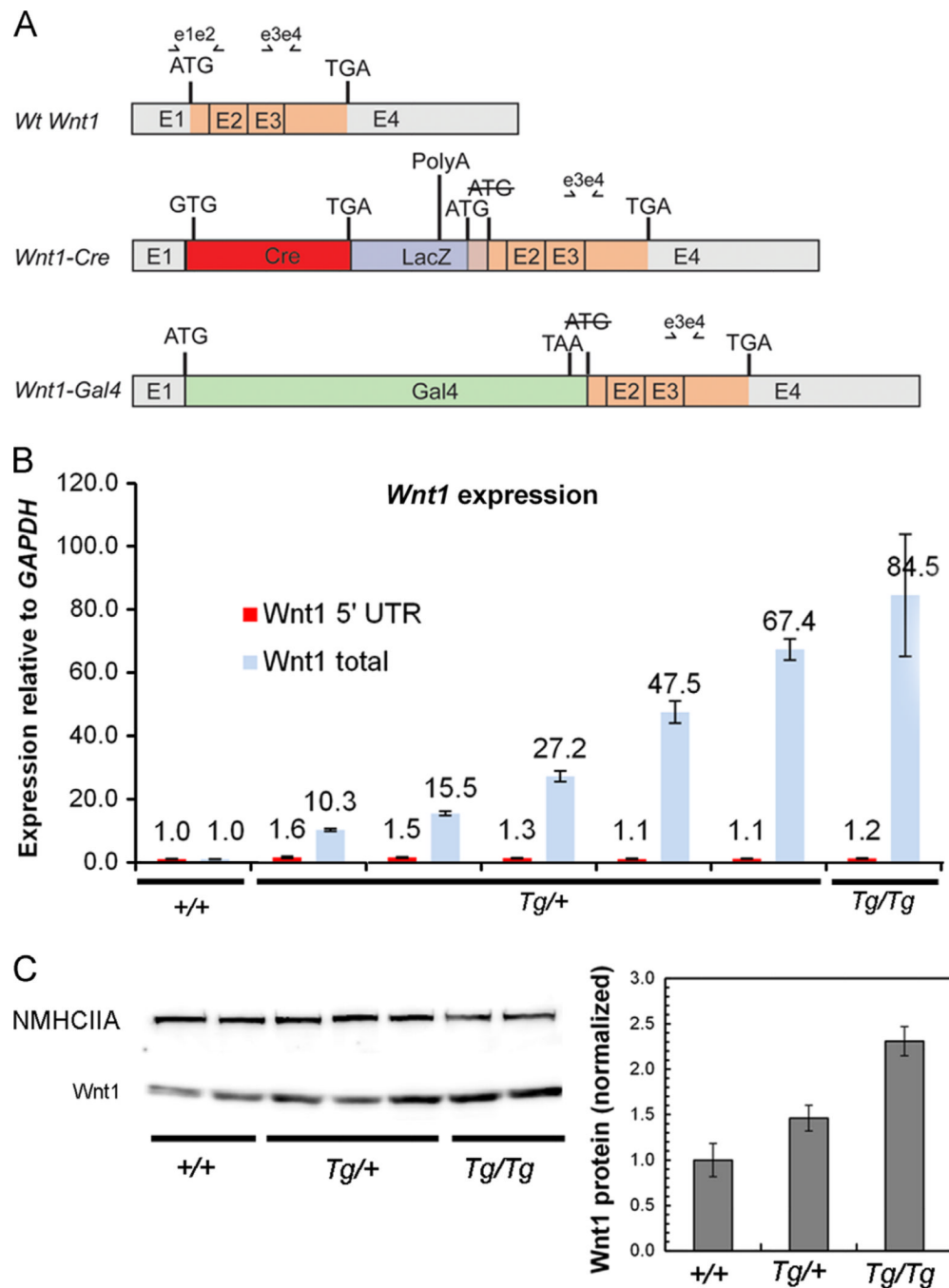


Fig. 2. Molecular basis for ectopic *Wnt1* expression in *Wnt1-Cre^{Tg}* embryos. (A) Schematic representation of *wild-type* *Wnt1* transcript and two *Wnt1-Cre^{Tg}*-specific *Wnt1* transcripts. A *Wnt1-Cre* chimeric transcript carrying the complete *Cre* open reading frame, followed by part of the *LacZ* gene, also has an open reading frame predicted to encode the entire *Wnt1* transcript plus 165 5 base-pairs derived from the *LacZ* sequence. The *Wnt1-Gal4* chimeric transcript is not predicted to encode a complete *Wnt1* open reading frame. (B) Quantitative RT-PCR using primers recognizing all three *Wnt1* transcripts (e3e4 in A) indicates variable overexpression of *Wnt1* transcript in E13.5 embryonic *Wnt1-Cre^{Tg}* heads. Quantitative RT-PCR using primers recognizing only the *wild-type* *Wnt1* transcript indicate that endogenous

Wnt1 expression remains similar between genotypes (e1e2 in A). Error bars represent standard deviation from qRT-PCR performed on individual embryos in triplicate. (C) Western blot analysis of Wnt1 from E14.5 embryonic head extracts reveals a 1.5-fold upregulation of Wnt1 protein in *Wnt1-Cre^{Tg}*⁺ embryos, and an approximately 2.3-fold upregulation of Wnt1 protein in *Wnt1-Cre^{Tg}*^{Tg} embryos. Non-muscle myosin heavy chain IIA was used as a loading control, and error bars represent standard error.

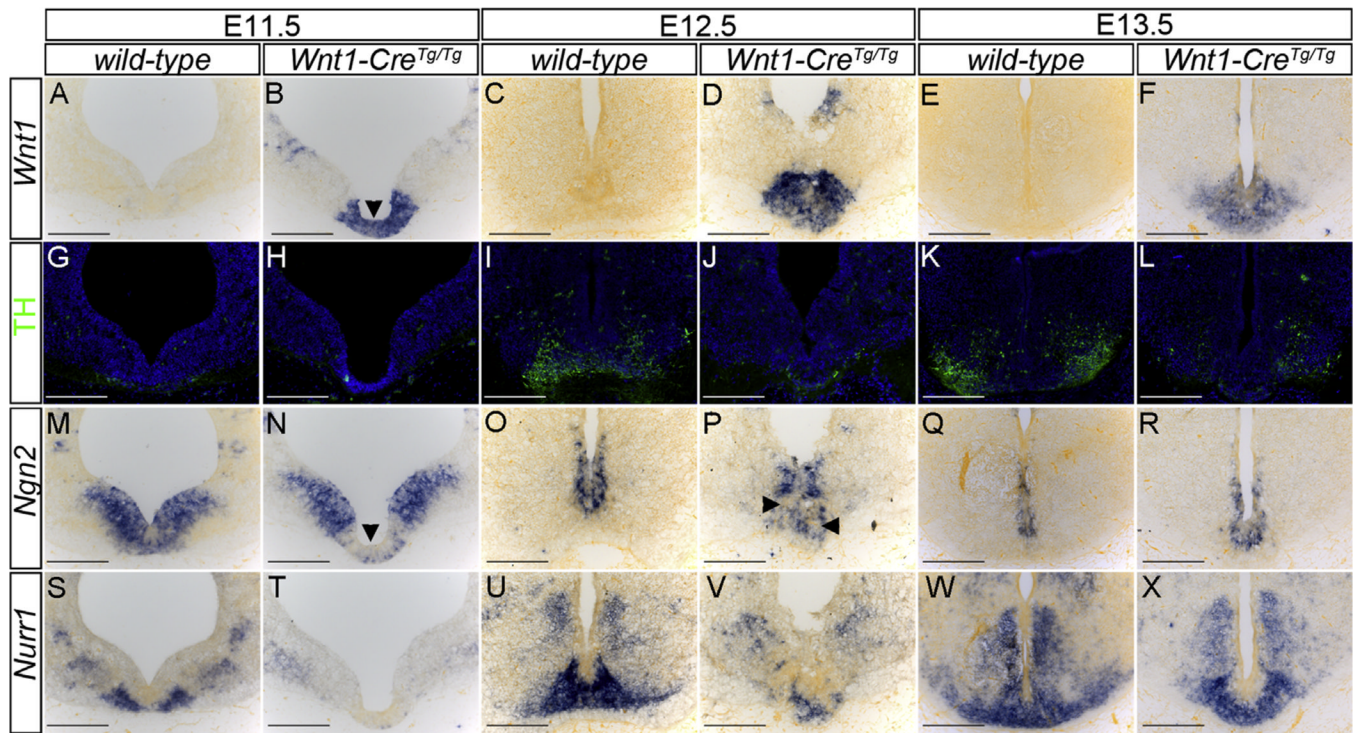


Fig. 3. Elevated *Wnt1* expression in the ventral midbrain disrupts the mDA differentiation program. (A–F) *In situ* hybridization of coronal sections of the ventral midbrain reveals a dramatic elevation in *Wnt1* expression in the ventral midbrain region of *Wnt1-Cre^{Tg/Tg}* embryos (B,D,F) compared with *wild-type* (A,C,E). (G–L) Immunostaining of sections adjacent to A–F with an antibody to tyrosine hydroxylase (TH) reveals a nearly complete absence of mDA neurons in E12.5 *Wnt1-Cre^{Tg/Tg}* embryos (J) compared to *wild-type* (I). Dramatically reduced TH staining is observed in E13.5 *Wnt1-Cre^{Tg/Tg}* embryos (L) compared to *wild-type* (K). (M–R) *In situ* hybridization of sections adjacent to A–F reveals the absence of expression of *Ngn2* within the *Wnt1* misexpression domain in E11.5 *Wnt1-Cre^{Tg/Tg}* embryos (arrowheads in B,N) compared to *wild-type* (M). At E12.5, reduced *Ngn2* expression is observed within this domain in *Wnt1-Cre^{Tg/Tg}* embryos (arrowheads in P) compared to *wild-type* (O), whereas *Ngn2* expression appears to have recovered at E13.5 (Q,R). *In situ* hybridization of sections adjacent to A–F reveals a complete loss of *Nurr1* expression in *Wnt1-Cre^{Tg/Tg}* embryos compared to *wild-type* at E11.5 (S,T) and E12.5 (U,V). Nearly complete recovery of *Nurr1* expression is observed at E13.5 (W,X). Scale bars: 200 μ m.

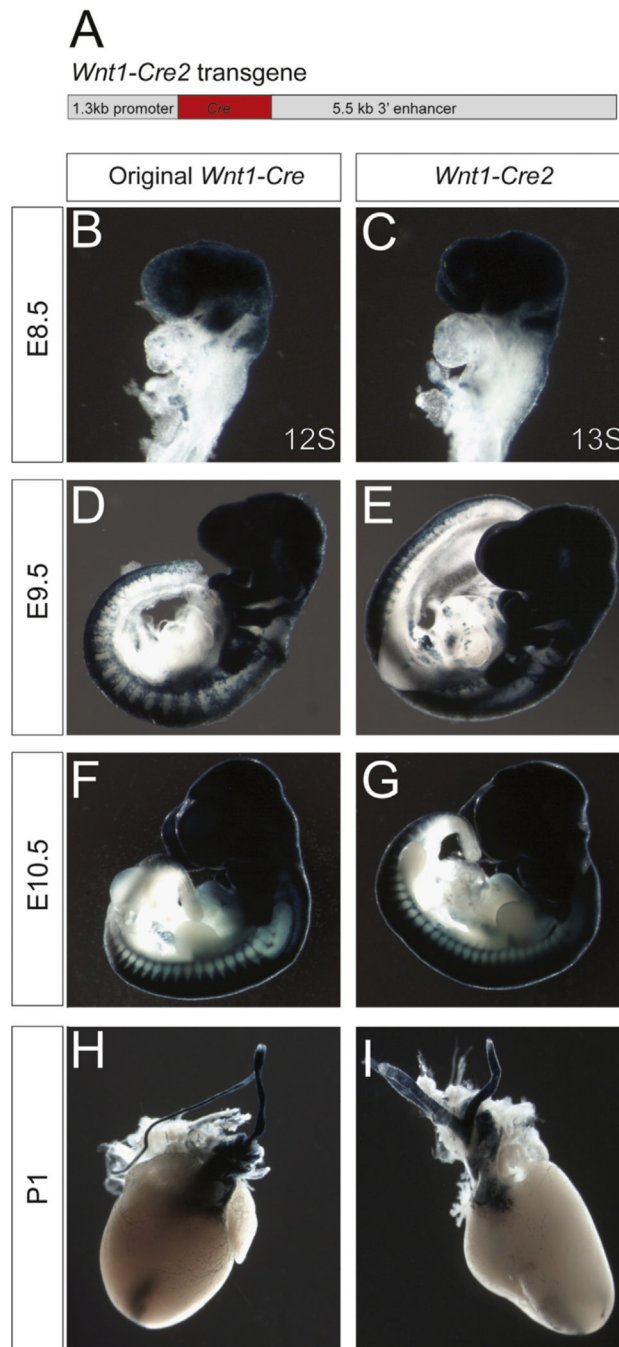


Fig. 4. The new *Wnt1-Cre2* transgenic mouse line mediates recombination in the neural crest. (A) Schematic representation of the new *Wnt1-Cre2* transgenic construct. (B–I) β -galactosidase staining of *Wnt1-Cre^{Tg+}; ROSA26R^{+/-}* embryos from the original line (B,D,F,H) compared with newly generated *Wnt1-Cre2^{Tg+}; ROSA26R^{+/-}* transgenic mice (C,E,G,I), in which the *Wnt1* coding sequence is not included in the transgene, at progressive stages of development. Comparable staining is observed in the neuroepithelium and migrating neural crest of E8.5 embryos in *Wnt1-Cre2^{Tg+}; ROSA26R^{+/-}* (C) compared to the original *Wnt1-Cre^{Tg+}; ROSA26R^{+/-}* (B). No overt difference was seen at E9.5 (D,E) or E10.5 (F,G), or in whole mount β -galactosidase staining of hearts at P1 (H,I).