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Excessive Wnt/beta–catenin signaling promotes midbrain floor plate neurogenesis, but results in vacillating dopamine progenitors

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Abstract

The floor plate (FP), a ventral midline structure of the developing neural tube, has differential neurogenic capabilities along the anterior-posterior axis. The midbrain FP, unlike the hindbrain and spinal cord floor plate, is highly neurogenic and produces midbrain dopaminergic (mDA) neurons. Canonical Wnt/beta–catenin signaling, at least in part, is thought to account for the difference in neurogenic capability. Removal of beta–catenin results in mDA progenitor specification defects as well as a profound reduction of neurogenesis.

To examine the effects of excessive Wnt/beta–catenin signaling on mDA specification and neurogenesis, we have analyzed a model wherein beta–catenin is conditionally stabilized in the

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Authors' contributions

N.N., M.J.P., and M.J. designed and conducted the experiments, prepared figures and participated in writing the manuscript. J.F.P. and A.A. helped to characterize the conditional mutants. M.T. provided the *Ctmb1lox(ex3)* mouse strain. Y.C.M. generated and provided the guinea-pig Lmx1a and Neurog2 antibodies. R.A. supervised the study and wrote the manuscript. All authors read and approved the final manuscript.

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Shh+ domain. Here, we show that the *Foxa2*+/*Lmx1a*+ domain is extended rostrally in mutant embryos, suggesting that canonical Wnt/beta-catenin signaling can drive FP expansion along the rostrocaudal axis. Although excess canonical Wnt/beta-catenin signaling generally promotes neurogenesis at midbrain levels, less tyrosine hydroxylase (Th)+, mDA neurons are generated, particularly impacting the Substantia nigra pars compacta. This is likely because of improper progenitor specification. Excess canonical Wnt/beta-catenin signaling causes downregulation of net *Lmx1b*, *Shh* and *Foxa2* levels in mDA progenitors. Moreover, these progenitors assume a mixed identity to that of *Lmx1a*+/*Lmx1b*+/*Nkx6-1*+/*Neurog1*+ progenitors. We also show by lineage tracing analysis that normally, *Neurog1*+ progenitors predominantly give rise to *Pou4f1*+ neurons, but not Th+ neurons. Accordingly, in the mutant embryos, *Neurog1*+ progenitors at the midline generate ectopic *Pou4f1*+ neurons at the expense of Th+ mDA neurons. Our study suggests that an optimal dose of Wnt/beta-catenin signaling is critical for proper establishment of the mDA progenitor character. Our findings will impact embryonic stem cell protocols that utilize Wnt pathway reagents to derive mDA neuron models and therapeutics for Parkinson's disease.

Keywords

midbrain dopaminergic neurons; floor plate; Wnt/beta-catenin; *Lmx1b*

Background

The midbrain floor plate (FP), but not the hindbrain or spinal cord FP, is highly neurogenic, and gives rise to midbrain dopaminergic (mDA) neurons (Andersson et al., 2006; Blaess et al., 2011; Bonilla et al., 2008; Hayes et al., 2011; Joksimovic et al., 2009a; Joksimovic et al., 2009b; Kittappa et al., 2007; Ono et al., 2007). The neurogenic character of the FP can be attributed to the presence of Wnts in the midbrain FP, but not in the hindbrain or spinal cord FP (Joksimovic and Awatramani, 2014). The role of Wnt signaling in the midbrain FP is thus significant and warrants further attention.

Several lines of evidence have suggested the importance of Wnts in midbrain FP neurogenesis and mDA neuron specification (Alves dos Santos and Smidt, 2011). *Wnt1* mutants display a drastic deficit in mDA neuron numbers (Prakash et al., 2006; Yang et al., 2013), which is exacerbated in the *Wnt1/Wnt5a* double mutant (Andersson et al., 2006). The conditional removal of beta-catenin results in alterations in mDA neuronal patterning and neurogenesis, and significantly less mDA neurons (Chilov et al., 2010; Joksimovic et al., 2009b; Tang et al., 2009). In these mutants, the midbrain FP is less proliferative, partly loses expression of *Otx2*, *Msx1*, *Neurog2* (Joksimovic et al., 2009b), and *Lmx1a* (Chilov et al., 2010; Joksimovic et al., 2009b), and maintains the expression of *Shh*, *Spon1* and *Lmx1b*, thereby molecularly resembling the hindbrain FP (Joksimovic et al., 2009b). In contrast, FP-specific stabilization of beta-catenin results in increased proliferation, the induction of *Lmx1a*, *Otx2*, *Msx1*, and *Neurog2*, and suppression of *Shh*, *Spon1* and *Lmx1b* in the hindbrain. In such mutants, the hindbrain and spinal cord were highly neurogenic, but ectopic mDA neurons were restricted to the rostral aspect of rhombomere 1 (Joksimovic et al., 2012; Joksimovic et al., 2009b).

Given the importance of Wnt/beta-catenin signaling for mDA specification and neurogenesis, and the tight spatiotemporal expression of *Wnt1* at the midline, we sought to analyze the effect of excessive Wnt/beta-catenin signaling on the ventral midline. We found that in *Shh::cre;Ctnnb1^{lox(ex3)}* embryos, the *Foxa2* and *Lmx1a* domains are rostrally extended along the anterior-posterior axis. At midbrain levels, fewer Th+ neurons were observed, particularly in the developing Substantia Nigra pars compacta (SNpc) region. We found that overall proliferation and neurogenesis are increased, and therefore are unlikely to account for reduced Th+ mDA neurons in this mutant. We next reasoned that this reduction of Th+ mDA neurons was because of perturbations in mDA progenitor gene expression profiles. We determined that stabilization of beta-catenin leads to net downregulation of key mDA neuron determinants, particularly *Lmx1b* and *Foxa2*, and failure to repress lateral markers such as *Nkx6-1* and *Neurog1* from the midline. We also show by genetic inducible fate mapping that *Neurog1+* progenitors normally give rise to *Pou4f1+* neurons of the red nucleus. Thus, excessive Wnt/beta-catenin signaling results in a vacillating mDA progenitor pool (*Lmx1a+/Lmx1b+/Nkx6-1+/Neurog1+*), that generates at least two distinct neuron populations: *Lmx1a+/Lmx1b+/Th+* and *Lmx1a+/Lmx1b+/Pou4f1+* neurons. These studies further uncover the molecular logic underlying mDA specification and neurogenesis, and reveal that Wnt/beta-catenin signaling needs to be carefully titred to maintain optimal mDA progenitor characteristics and generate the correct numbers of mDA neurons.

Results

Stabilization of beta-catenin in the *Shh* domain results in excessive and ectopic Wnt/beta-catenin signaling

We first performed experiments to confirm that *Shh::cre;Ctnnb1^{lox(ex3)}* embryos demonstrated excessive Wnt/beta-catenin signaling in the FP. We generated *Shh::cre;Ctnnb1^{lox(ex3)}, Axin2^{lacZ}* embryos in which a *NLS-lacZ* reporter gene is expressed under the control of the *Axin2* promoter region. *Axin2* is a direct target of the Wnt/beta-catenin pathway and serves as an indicator of canonical Wnt/beta-catenin signaling (Jho et al., 2002; Lustig et al., 2002; van Amerongen et al., 2012). In the midbrain FP, X-gal labeling is markedly increased in *Shh::cre;Ctnnb1^{lox(ex3)}, Axin2^{lacZ}* compared to *Axin2^{lacZ}* controls (Figure 1A and C). In the hindbrain FP, where X-gal labeling is largely absent in controls (Figure 1B), prominent X-gal labeling is observed in mutants (Figure 1D). In the rostral hypothalamus, a marked increase in X-gal labeling is also observed in mutants (Supplemental Figure 1A and B). Thus, *Shh::cre;Ctnnb1^{lox(ex3)}* embryos demonstrate excessive Wnt/beta-catenin signaling throughout the *Shh* domain.

Ectopic Wnt/beta-catenin signaling results in a rostral expansion of the *Foxa2* domain

In normal embryos, the *Foxa2+/Lmx1a+* expression domain stops in a sharp boundary rostral to base of the zona limitans intrathalamica (ZLI). In contrast, *Shh* expression extends more rostrally, in a manner that is dependent on specific rostral enhancers (Geng et al., 2008; Jeong et al., 2006). In *Shh::cre;Ctnnb1^{lox(ex3)}* embryos, Wnt/beta-catenin signaling is increased throughout the *Shh* domain (Figure 1C and D; Supplemental Figure 1B). In sagittal sections of *Shh::cre;Ctnnb1^{lox(ex3)}* embryos, we observed a marked rostral expansion of *Foxa2* beginning at E10.5 (Supplemental Fig. 2A, A', B, and B') and persisting

through E12.5, where *Foxa2* expression extends to the anterior hypothalamus (Figure 2B, B' Bottom arrowhead, and C; Supplemental Figure 1D). Using the FP/ZLI border as a landmark (Figure 2A' and B' top arrowheads), we measured the distance from the ZLI to the rostral *Foxa2* boundary (Figure 2A' and B' bottom arrowheads). Our measurements revealed a significant expansion of the rostral *Foxa2* domain between the controls and mutants (Figure 2C). Likewise, at least by E12.5, the *Lmx1a* domain is extended rostrally, although at reduced levels in *Shh::cre;Ctnnb1lox(ex3)* embryos (Figure 2B''). These results demonstrate that Wnt/beta-catenin signaling could potentially drive the expansion of the *Foxa2*+/*Lmx1a*+ domain along the rostro-caudal axis.

Sustained Wnt/beta-catenin signaling results in fewer mDA neurons

To determine whether excessive canonical Wnt/beta-catenin signaling affects mDA neuron production, we examined expression of multiple mDA neuron markers in *Shh::cre;Ctnnb1lox(ex3)* midbrain sagittal and coronal sections at E12.5, E14.5, and E18.5. Our initial analyses at E12.5 and E14.5 revealed a drastic reduction of Th in mutants compared to controls (Figures 2A and B; Fig. 3A and B; Supplemental Fig. 3E and F). This effect was especially pronounced in rostral midbrain sections (Figure 3E), where we observed up to a 75% reduction in Th+ neuron numbers. We also observed a reduction in the number of cells expressing *Slc6a3* and *Pitx3* (Supplemental Fig. 3A–F) in *Shh::cre;Ctnnb1lox(ex3)* embryos. Furthermore, *Slc6a3* expression appears lower even in the remnant Th+ neurons (Supplemental Fig. 3E and F). At E18.5, fewer Th+ neurons are observed in the developing SNpc of mutants compared to controls, consistent with a previous study (Tang et al., 2010). In contrast, the Ventral Tegmental Area (VTA) does not appear drastically diminished (Figure 3D). Some ectopic Th+ neurons are also observed in the interpeduncular region, likely representing ectopic hindbrain FP derived mDA neurons (Joksimovic et al., 2012). Together, these results demonstrate that excessive Wnt/beta-catenin signaling leads to reduced mDA neuron numbers.

Excessive Wnt/beta-catenin signaling promotes neurogenesis

We next sought to determine if the reduction in Th+ neurons in *Shh::cre;Ctnnb1lox(ex3)* was the result of changes in mDA proliferation or neurogenesis. To assess the effect of Wnt/beta-catenin signaling on mDA progenitor proliferation and cell cycle exit, we performed short and long pulse BrdU analyses. First, we investigated whether stabilizing beta-catenin may selectively affect the fraction of progenitors in S-phase within either the *Lmx1a*+ or *Nkx6-1*+ domains. To accomplish this, we determined the Labeling Index (Omodei et al., 2008) at E10.5 and E12.5 by providing a short 30 minute BrdU pulse and measuring the percentage of BrdU+ cells over the total number of cells within either of the two domains. We found no differences in the fraction of progenitors in S-phase at E10.5 in either domain between control and mutant embryos (Figure 4A–C; percentage of BrdU+/DAPI+ in *Lmx1a* domain and BrdU+/DAPI+ in *Nkx6-1* domain; n=3 each; DAPI not shown). However at E12.5, 30 minute BrdU labeling revealed an increase in the fraction of progenitors in S-phase within the *Lmx1a* domain in *Shh::cre;Ctnnb1lox(ex3)* when compared to controls (Figure 4D–F;). Concurrently at E12.5, a significant increase (p<0.001) in the total number of BrdU+ cells per section was observed in mutants (n=3; 284 ± 28 s.e.m) when compared to controls (n=3; 135 ± 10 s.e.m), consistent with previous observations that Wnt/beta-

catenin signaling promotes progenitor proliferation (Joksimovic et al., 2009b; Tang et al., 2010).

We also measured the net amount of neurogenesis by performing 24 hour BrdU pulse labeling experiments. In these experiments, the total number of cells that have exited the cell cycle between E12.5 and E13.5 (BrdU+;Ki67-) is significantly increased in the mutants (Figure 4G-I). The quit fraction is minimally altered at this stage when measured as BrdU+;Ki67-/BrdU+ total (control 0.646 ± 0.016 s.e.m.; mutant 0.626 ± 0.001 s.e.m.; $p=0.380$) or when measured similar to Tang et al. as BrdU+;Ki67-/BrdU+;Ki67+ (control 2.37 ± 0.275 s.e.m.; mutant 1.88 ± 0.035 s.e.m.; $p=0.208$). Thus, we observe more neurons exiting the cell cycle between E12.5 and E13.5 likely due to an increase in the number of progenitors at E12.5, but not due to an increase in the quit fraction. Finally, by E15.5, large numbers of Lmx1a+ cells can be seen in the mantle zone in mutants compared to controls (Figure 4J and K). Quantification of Lmx1a+ cells revealed a ~57% increase in mutants compared to controls, consistent with increased FP proliferation and neurogenesis (Figure 4L). This increased proliferation and neuronal production are consistent with a role for Wnt/beta-catenin signaling in promoting FP neurogenesis (Joksimovic et al., 2009b; Tang et al., 2010). Together, these data show that the reduction in numbers of Th+ neurons in *Shh::cre;Ctnnb1^{lox(ex3)}* embryos is not due to a general decrease in FP neurogenesis.

Excessive Wnt/beta-catenin signaling results in vacillating mDA progenitors

We next investigated whether the reduction in Th+ neurons in *Shh::cre;Ctnnb1^{lox(ex3)}* mutants was due to disruptions in progenitor gene expression profiles. We surveyed the expression of key genes in mDA progenitors and neighboring ventral midbrain progenitor domains. First, we screened for early expression of *Foxa2* and *Shh* which are known to be critical genetic networks for the regulation of mDA neuron development (Blaess et al., 2006; Chung et al., 2009; Ferri et al., 2007; Metzakopian et al., 2012; Perez-Balaguer et al., 2009). Although there is significant ectopic expansion of *Foxa2* expression in the hypothalamus (Figure 2A', B' and C; Supplemental Figure 2A-B'), there is an apparent reduction of *Foxa2* expression in the mDA ventricular zone (Figure 5A and B, Supplemental Figure 4A and B) consistent with a previous study (Tang et al., 2010). Similarly, we found that *Shh* is downregulated in the mutant ventral midbrain compared to the control which is consistent with our previous study (Figure 5C and D; Joksimovic et al., 2009b). Another key FP gene *Spon1* is also downregulated in the mutant ventricular zone (Figure 5E and F).

We then examined other factors known to be expressed within the mDA progenitor domain and important in mDA development. In the control midbrain, *Lmx1b* starts to be downregulated in mDA progenitors at ~E11.0 in a rostral-to-caudal manner (Anderegg et al., 2013; Andersson et al., 2006). However, *Lmx1a* does not appear to be significantly reduced at this time point. In E10.5 and E11.5 mutants, we observed that at the midbrain ventral midline, *Lmx1b* is prematurely downregulated relative to controls, whereas *Lmx1a* was not drastically altered (Figure 6A, B, K, L, M, and N). *Lmx1b* was also reduced in the hindbrain and spinal cord FP (Joksimovic et al., 2012). *Wnt1*, *Wnt5a*, and *Aldh1a1* also appeared reduced in mutants compared to controls (Figure 6C-H). Neurog2+ cells were increased in

mutants (Figure 6Q and R), consistent with the previously observed increase in FP neurogenesis (Figure 4).

We next analyzed genes that are known to be expressed adjacent to the mDA progenitor domain. *Neurog1* and *Nkx6-1*, which are adjacent to, but largely excluded from the *Lmx1a/Lmx1b* domain in controls (Figure 6I, M and O), appear within the *Lmx1a/Lmx1b* domain in mutants (Figure 6J, N and P); in the case of *Neurog1*, this is most evident in rostral midbrain sections. Ectopic midline *Nkx6-1* expression is prominent at E10.5 and E11.5 (Figure 6J and N) but not at later time points (data not shown).

Our previous studies have shown that canonical Wnt/beta-catenin signaling is required for *Lmx1a* expression in mDA progenitors (Joksimovic et al., 2009b). Additionally, increasing Wnt/beta-catenin signaling in the hindbrain FP was sufficient to induce *Lmx1a* expression in this region (Joksimovic et al., 2012). Thus it was interesting that in the *Shh::cre;Ctnnb1lox(ex3)* mutants, *Lmx1a* was not induced in the *Nkx6-1* domain despite the presence of *Foxa2*, and increased Wnt signaling in this region. We postulated that in addition to *Foxa2* and canonical Wnt signaling, *Lmx1b* was also required for the induction of *Lmx1a* expression. We therefore overexpressed *Lmx1b* (Figure 7B) in the ventral midbrain using a *Shh::Cre;Lmx1bOE* model (Li et al., 2010). In these embryos we observed a significant lateral expansion of *Wnt1*, although at the midline *Wnt1* remained absent similar to controls (Figure 7C and D). Additionally, the *Lmx1a* domain was expanded well into the *Nkx6-1* domain in mutants but not in controls (Figure 7E and F). Our interpretation of this data is that in addition to Fox genes (Ferri et al., 2007) and canonical Wnt signaling (Joksimovic et al., 2009b), *Lmx1b* is required for the induction of *Lmx1a*. This is consistent with the finding that *Lmx1b* binds to regions upstream of *Lmx1a* (Chung et al., 2009).

Pou4f1+ neurons are derived from Neurog1+ progenitors

Having observed *Neurog1* expanded beyond its lateral domain into the midline in *Shh::cre;Ctnnb1lox(ex3)* mutants (Figure 6P), we sought to determine the fate of ventral midbrain *Neurog1+* progenitors in normal embryos. To do this, we performed a fate-mapping experiment by crossing a *Neurog1::creERT2* mouse to *Ai9*, a *Rosa26-tdTomato* reporter line. Females harboring *Neurog1::creERT2,Ai9* embryos were injected with tamoxifen at E9.75 and harvested at E12.75. In these embryos, several *Pou4f1+/tdTomato+* cells were observed immediately lateral to the largely *Pou4f1-/tdTomato-mDA* neuron domain (Figure 8B–F). This data suggests that *Neurog1+* progenitors give rise to *Pou4f1+* red nucleus neurons consistent with our previous fate maps and models proposed by others (Blaess et al., 2011; Joksimovic et al., 2009a; Nakatani et al., 2007; Prakash et al., 2009).

Pou4f1+ neurons are generated from the ventral midline in *Shh::cre;Ctnnb1lox(ex3)* mutant embryos

Considering the fact that *a)* *Nkx6-1* is required for generation of *Pou4f1+* neurons (Prakash et al., 2009) *b)* *Neurog1+* progenitors normally give rise to *Pou4f1+* neurons (Figure 8) and *c)* *Nkx6-1* and *Neurog1* are expressed in midline progenitors in mutants (Figure 6J and P), we next examined the expression of *Pou4f1* in *Shh::cre;Ctnnb1lox(ex3)* embryos. Consistent with ectopic expression of *Nkx6-1* and *Neurog1* in the mutant mDA progenitor region, we

detected large numbers of medially located Pou4f1+ cells in the mantle zone, particularly in rostral sections (Figure 9B, D, F, G, I, K, and M; Supplemental Fig.5C, D, C', and D'). Most of these Pou4f1+ neurons express Lmx1b (Figure 9D) and Lmx1a (Figure 9K, U), suggesting that they are derived from the Lmx1a/Lmx1b mDA progenitor domain. Some of these ectopic Pou4f1+ neurons also expressed Lhx1, a marker normally not observed in midline regions (Supplemental Fig.5C' and D'). In controls, a few Pou4f1+ cells were also medially located in the mantle zone predominantly at the rostral midbrain levels consistent with previous studies (Lahti et al., 2012). Few, if any, Pou4f1+ cells co-expressed Th in mutants (Figure 9F inset and Figure 9M and U; Supplemental Fig. 6B). Analysis of E15.5 embryos also revealed persistence of Pou4f1+ cells in ectopic medial locations in mutants compared to controls (data not shown). These results, together with recent studies (Andersson et al., 2006; Nakatani et al., 2010) suggest that depression of net Lmx1b/Foxa2 and possibly other factors by Wnt/beta-catenin signaling, prevents robust antagonism of *Nkx6-1* and *Neurog1* at the midline; this results in a mixed Lmx1a/Lmx1b+/Nkx6-1+/Neurog1+ progenitor pool and the genesis of both Th+ and Pou4f1+ neurons from the Lmx1a/Lmx1b progenitor domain.

Discussion

We show here that although Wnt/beta-catenin signaling increases the number of neurons produced from the Lmx1a domain, this excessive signaling results in gene expression changes in mDA progenitors that, in part, account for a decrease in numbers of Th+ mDA neurons. By examining gene expression in the mDA progenitor region, we find that excessive Wnt/beta-catenin signaling results in an ambivalent progenitor pool that generates both Th+ and Pou4f1+ neurons. The data presented here could have relevance for optimizing embryonic stem cell protocols that utilize Wnt pathway reagents to derive mDA neurons (Cajanek et al., 2009; Chung et al., 2009; Cooper et al., 2010; Fasano et al., 2010; Grealish et al., 2014; Kirkeby et al., 2012; Kriks et al., 2011; Xi et al., 2012).

Here, we report that excessive Wnt/beta-catenin signaling reduces the number of Th+ mDA neurons. In a previous study (Tang et al., 2010), this reduction in *Shh::cre; Ctnnb1^{lox(ex3)}* mutants was partly attributed to a reduced rate of cell cycle exit between E11.5 and E12.5. The authors also reported increased proliferation at E12.5 and no detectable increase in cell death. Our data agree with these findings, in that the total number of Lmx1b+ postmitotic mDA neurons appears to be slightly reduced at E12.5 (Figure 9D), consistent with a decreased quit fraction between E11.5 and E12.5 (Tang et al., 2010). We also observed increased proliferation at E12.5. However, we extended this analysis by showing that between E12.5 and E13.5, there was an increase in the total numbers of postmitotic, mDA lineage neurons produced (Figure 4). In fact, by E15.5, the overall number of Lmx1a+ post mitotic neurons produced from the mDA domain was increased (Figure 4L). To reconcile these studies, we propose a model in which excessive Wnt/beta-catenin signaling initially increases proliferation of mDA progenitors and initially reduces the rate of cell cycle exit. As development proceeds, the surplus of accumulated mDA progenitors exits the cell cycle and produces excess Lmx1a+ neurons. Therefore, although excessive Wnt/beta-catenin signaling initially reduces cell cycle exit (Tang et al., 2010), it promotes overall neurogenesis (this study). Furthermore, since there is in fact an increase in Lmx1a+ mDA

lineage neuron production, alterations in neurogenesis cannot explain the observed reduction of total Th⁺ neurons in these mutants.

Why are fewer Th⁺ neurons observed in the ventral midbrain of *Shh::cre;Ctnnb1^{lox(ex3)}* mutants? During normal development, key mDA neuron determinants such as *Lmx1b*, *Foxa2*, and possibly other factors are required for repressing lateral, predominantly non-mDA progenitor fates (Andersson et al., 2006; Deng et al., 2011; Nakatani et al., 2010). Specifically, in the Nakatani et al. study, only transgenes containing both *Foxa2* and *Lmx* genes were able to suppress *Neurog1* expression. Thus, we postulate that downregulation of these key mDA genes at the ventral midline in *Shh::cre;Ctnnb1^{lox(ex3)}* mutants likely results in an inability to repress the lateral progenitor program. This causes lateral markers such as *Nkx6-1* and *Neurog1* to be ectopically expressed at the ventral midline within the mDA progenitor pool. As a result, the mDA progenitor pool takes on a hybrid molecular profile comprising of both mDA and lateral progenitor markers (*Lmx1a/Lmx1b*+/*Nkx6-1*+/*Neurog1*+), rendering it bipotent. In normal embryos, *Neurog1* progenitors give rise to *Pou4f1*+ cells. Accordingly, in the *Shh::cre;Ctnnb1^{lox(ex3)}* mutants, *Pou4f1*+ cells are observed at the midline at the expense of Th⁺ cells. These *Pou4f1*+ cells co-express *Lmx1a/Lmx1b*, indicating their origin in the mDA progenitor domain. Thus, we posit that by down regulating *Lmx1b* and *Foxa2*, and potentially other unknown factors, excessive Wnt/beta-catenin signaling results in a vacillating progenitor phenotype (*Lmx1b^{low}*, *Foxa2^{low}*, *Lmx1a*+, *Nkx6-1*+, *Neurog1*+) that gives rise to many *Pou4f1*+ neurons at the expense of Th⁺ neurons. It remains to be determined whether these *Pou4f1*+ neurons persist.

Excess numbers of *Pou4f1*+ neurons have also been observed at the midline of FGF receptor loss-of-function mutants. In such mutants, early DA precursors are intermingled with *Pou4f1/Lmx1a/FoxP1*+ cells in the ventral midbrain (Lahti et al., 2012). In addition these mutants, show decreased expression of *Wnt1*, *Wnt5a*, and *Aldh1a1* in the midbrain. The similarities between the *Shh::cre;Ctnnb1^{lox(ex3)}* mutants, and the FGFR mutants open the possibility that Wnt/beta-catenin signaling and FGF signaling have an antagonistic interaction, which should be further investigated.

In *Shh::cre;Ctnnb1^{lox(ex3)}* mutants, we find a more severe reduction in the SNpc compared to the VTA, consistent with the Tang et al study. Why might this be? One possibility is alterations in the timing of neurogenesis at E11.5 (Tang et al., 2010), a time when substantial SNpc neurons are born (Bye et al., 2012). Additionally, even at early embryonic ages, rostral regions are much more severely affected, compared to caudal regions. More *Pou3f1* neurons are produced rostrally, instead of Th⁺ neurons. Since the SNpc is likely derived from rostral DA progenitors (Blaess et al., 2011; Hayes et al., 2011; Joksimovic et al., 2009a; Veenvliet et al., 2013), a more severe loss of SNpc neurons is therefore a predictable finding. Why rostral DA progenitors are more severely affected, however remains to be determined. It is interesting to note, that Wnt signaling displays a rostral^{low} caudal^{high} gradient (Awatramani, unpublished results); therefore rostral SNpc progenitors may be more susceptible to elevation of Wnt signaling. Understanding the establishment and functional consequences of Wnt gradients remains to be further studied.

In summary, we have demonstrated that excessive Wnt/beta-catenin signaling results in rostral expansion of the Foxa2+/Lmx1a+ domain, suggesting that canonical Wnt signaling can drive FP expansion. Further, we have shown that excessive Wnt/beta-catenin signaling in the midbrain FP increases neuron production. Despite its pro-neurogenic capabilities, excessive Wnt/beta-catenin signaling downregulates key mDA determinants, including Foxa2 and Lmx1b, and thus alters the mDA progenitor identity to that of Lmx1b^{low}, Foxa2^{low}, Lmx1a+, Nkx6-1+, Neurog1+ progenitors. These vacillating progenitors give rise to Pou4f1+ neurons, and Th+ mDA neurons. These data suggest that Wnt/beta-catenin signaling levels need to be fine-tuned to achieve the proper molecular signature of mDA progenitors. In this regard, *miR135a2*, which is predicted to target multiple aspects of the Wnt cascade, may play an important role (Anderegg et al., 2013). Further studies will be required to decipher the modulation of the Wnt pathway, the targets of canonical Wnt/beta catenin signaling and potential interactions with FGF signaling, as well as the potential impact of Wnt/beta catenin signaling on the genesis of diverse mDA neuron subtypes (Di Salvio et al., 2010; Panman et al., 2014; Poulin et al., 2014; Veenvliet et al., 2013) - these will be important for optimally deriving mDA neurons from stem cells, a key goal for Parkinson's disease modelling and therapeutics (Steinbeck and Studer, 2015).

Methods

Mouse strains and genotyping

Mice were maintained and sacrificed according to the protocols approved by the Northwestern University Animal Care and Use Committee. Male mice bearing a *GFPcre* fusion that was knocked in to the *Shh* locus (hereafter designated *Shh::cre*; (Harfe et al., 2004) were either crossed to female *Ctnnb1^{lox(ex3)}* female mice (Harada et al., 1999) to obtain *Shh::cre;Ctnnb1^{lox(ex3)}* embryos or to mice harboring the *Rosa26^{Lmx1b/+}* allele (Li et al., 2010). For fate mapping experiments, male mice bearing an *Ngn1::creER^{T2}* (Kim et al., 2011) allele were crossed to *Ai9* female reporter mice (Madisen et al., 2010). 2 mg of tamoxifen per 40 g of body weight were injected intraperitoneally into pregnant females to induce cre activity. The morning of the day when a vaginal plug was detected was designated as E0.5.

RNA in situ hybridization, X-gal, and fluorescent immunohistochemistry

Embryos or whole brains were harvested and fixed in 0.2%–4% PFA in PBS for various amount of time depending upon embryonic ages, and sectioned at 20–40 μ m. X-gal, immunohistochemistry, and *mRNA in situ* hybridization were performed as described (Joksimovic et al., 2009b). For fluorescent immunohistochemistry, tissue sections were post-fixed in 1%–4% PFA in PBS, rinsed in PBS, microwave treated with Antigen Unmasking Solution (Vector Laboratories), blocked in 5% donkey serum and 0.1% Triton X-100 in PBS, and incubated overnight at 4°C with primary antibodies diluted in blocking solution: rabbit Lmx1a (gift from Dr. Michael German; 1:1,000), guinea pig Lmx1a (1:20,000), rat BrdU (Serotec; 1:400), mouse Ki67 (BD Pharmingen; 1:200), guinea pig Lmx1b (gift from Dr. Thomas Muller and Dr. Carmen Birchmeier; 1:10,000), rabbit Lmx1b (custom made; 1:5,000), mouse Nkx6-1 (Developmental Studies Hybridoma Bank; 1:100), goat Foxa2 (Santa Cruz; 1:50), rat Slc6a3 (Santa Cruz; 1:50), goat anti Lhx1 (Santa Cruz; 1:50), Mouse

Isl1 (DSHB; 1:100), guinea pig Neurog2 (1:10,000), rabbit Pou4f1 (gift from Dr. Eric Turner; 1:4,000), mouse Pou4f1 (Santa Cruz; 1:100), rabbit and sheep Th (Pel Freeze; 1:500 and 1:250, respectively), and mouse HuC/D (Molecular Probes; 1:100). Sections were rinsed in PBS and incubated with appropriate Alexa 488, 555, and 647 (Molecular Probes) or Cy3 and Cy5 (Jackson ImmunoResearch) secondary antibodies diluted 1:250 in blocking solution, rinsed in PBS, covered with DAPI (1 mg/mL; Sigma) in PBS, rinsed in PBS, and coverslipped followed by epifluorescent (Leica) or confocal microscopy (Zeiss LSM 510 META and Leica DM6000B). Images were processed in Adobe Photoshop CS2 and CS5.

BrdU labeling/detection

Pregnant dams were injected with BrdU (50 mg/Kg, Sigma) and harvested either 30 minutes or 24 hours later. BrdU immunostaining was performed as described above.

Statistical analysis

All results were expressed as mean \pm standard error of the mean (s.e.m.) and analyses were performed using Microsoft Excel software. Two experimental groups were compared with Student's two sample *t* test. Significant differences were taken at *p* 0.05. For the 30 minute BrdU pulse, a number of BrdU+ cells within the Lmx1a+ ventricular zone were counted on every third 20 μ m section throughout the midbrain. For Pou4f1, Th, and Lmx1a quantification, the cells were counted within the dopaminergic field on every third 20 μ m section throughout the midbrain. To quantify the mDA progenitor cell cycle exit, BrdU was injected in pregnant mothers at E12.5 and embryos harvested at E13.5. The total number of BrdU+ cells and the number of BrdU+/Ki67 progenitors in the ventricular zone were counted within the Lmx1a domain on every third 20 μ m section throughout the midbrain. Similar to Tang et al., mDA progenitor cell cycle exit was also measured by counting the number of BrdU+/Ki67- in the mantle zone over the number of BrdU+/Ki67+ cells in the ventricular zone of the Lmx1a domain on every third 20 μ m section throughout the midbrain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- A mix of DA and red nucleus progenitors.
- Increase in proliferation and neurogenesis.
- Loss of SNpc population vs. VTA.

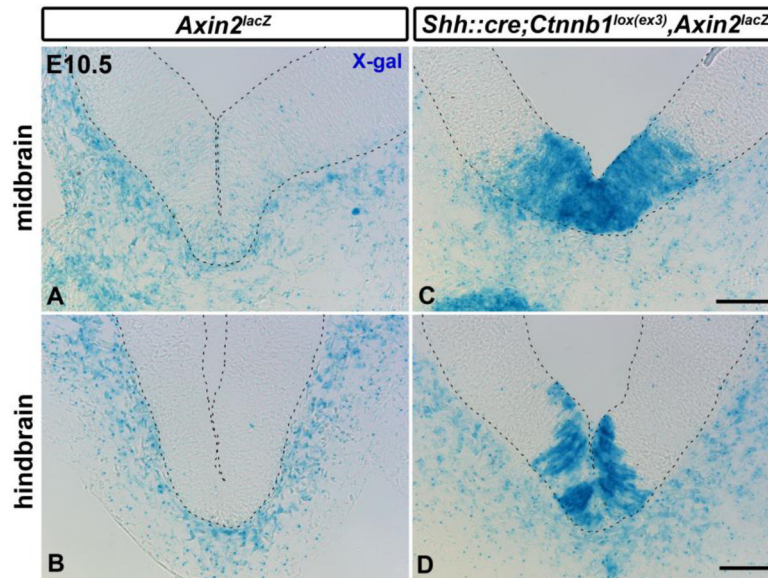


Figure 1. Stabilization of beta-catenin in the Shh+ midbrain and hindbrain floor plate results in drastically increased Wnt/beta-catenin signaling

(A–D) E10.5 coronal midbrain (A and C) and hindbrain (B and D) sections were labeled with X-gal to determine *Axin2^{lacZ}* expression in control (*Axin2^{lacZ}*; A and B) and mutant (*Shh::cre;Ctnnb1^{lox(ex3)},Axin2^{lacZ}*; C and D) embryos. (A and C) In the ventral midbrain, intense X-gal+ labeling is present in the mutant compared to the moderate X-gal labeling in the control. (B and D) In the ventral hindbrain, the mutant floor plate is characterized by a substantial number of Xgal+ cells in comparison to the corresponding region in the control, which has few to no Xgal+ cells. Black dotted lines delineate the midbrain and hindbrain parenchyma. mesenchymal cells surrounding the neural tube are Xgal+ in all sections shown. Scale bars: 50µm.

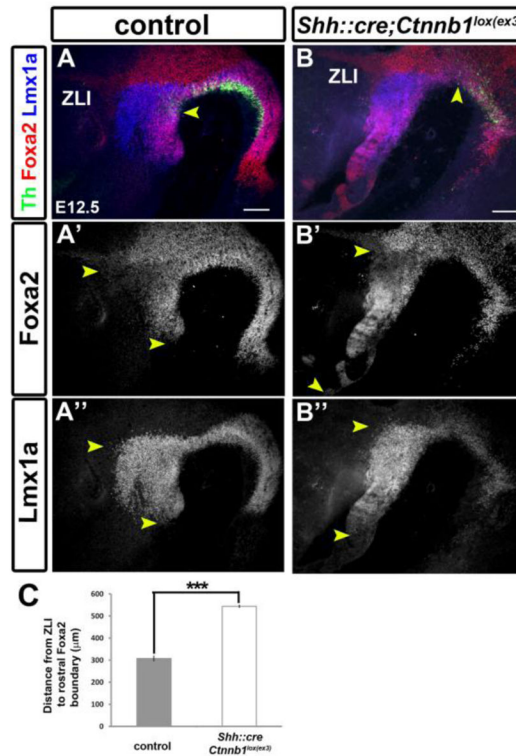


Figure 2. Sustained Wnt/beta–catenin signaling results in a rostral expansion of the Foxa2 domain

(A–C) E12.5 sagittal sections from control and *Shh::cre;Ctnnb1^{lox(ex3)}* mutant embryos were co-labeled with Th (A and B), Foxa2 (A, A', B, and B'), Lmx1a (A, A'', B, and B''). Mutant sections show an expanded diencephalic ventricle (A and B; see also Suppl. Fig 1). The number of Th+ mDA neurons (green) are severely reduced, particularly in rostral regions in the mutant (arrowheads) when compared to control. (A and B). Foxa2 expression extends rostrally, to the anterior hypothalamus (B and bottom arrowhead in B') in contrast to the sharp Foxa2 limit in the posterior hypothalamus in controls (A and bottom arrowhead in A'). (A, A'', B and B'') In mutants, similar to the anterior shift of Foxa2, Lmx1a expression extends rostrally (B and bottom arrowhead in B'') albeit at reduced levels. (C) A significant rostral expansion ($p < 0.001$) of the Foxa2 domain is observed in mutants ($544\mu\text{m} \pm 4.62$ s.e.m.) compared to controls (control $308\mu\text{m} \pm 10.6$ s.e.m.) when measured from the ZLI/FP boundary (Arrowheads in A' and B'). ZLI, zona limitans intrathalamica; FP, floor plate. Anterior is to the left, posterior to the right, dorsal up, and ventral down. Scale bars: $100\mu\text{m}$ in A and B.

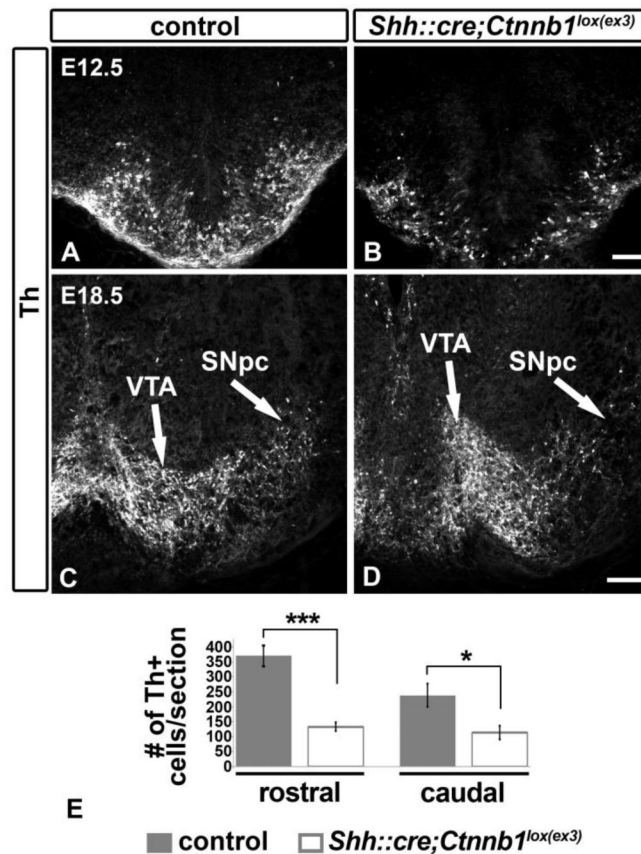


Figure 3. Constitutively active Wnt/beta-catenin signaling results in a severe reduction in numbers of Th+ neurons

E12.5 (A and B) and E18.5 (C and D) coronal midbrain sections were labeled with Th antibody in control (A and C) and *Shh::cre;Ctnnb1^{lox(ex3)}* mutant (B and D) embryos. (A and B) Note an apparent loss of Th+ cells in the mutant compared to the control at E12.5. (C and D) At E18.5, the developing Substantia Nigra pars compacta (SNpc; right arrows) is particularly affected, but the developing Ventral Tegmental Area is less affected (VTA; left arrows). (E) A significant decrease in the total number of Th+ cells per section at E12.5 is observed rostrally ($p < 0.001$) and caudally ($p < 0.05$) in the mutants ($n = 3$; rostral $133 \pm \text{s.e.m } 15$; caudal $114 \pm \text{s.e.m } 22$) in comparison to the control ($n = 3$; rostral $370 \pm \text{s.e.m } 35$; caudal $238 \pm \text{s.e.m } 39$). Scale bars: $50 \mu\text{m}$ in A–D.

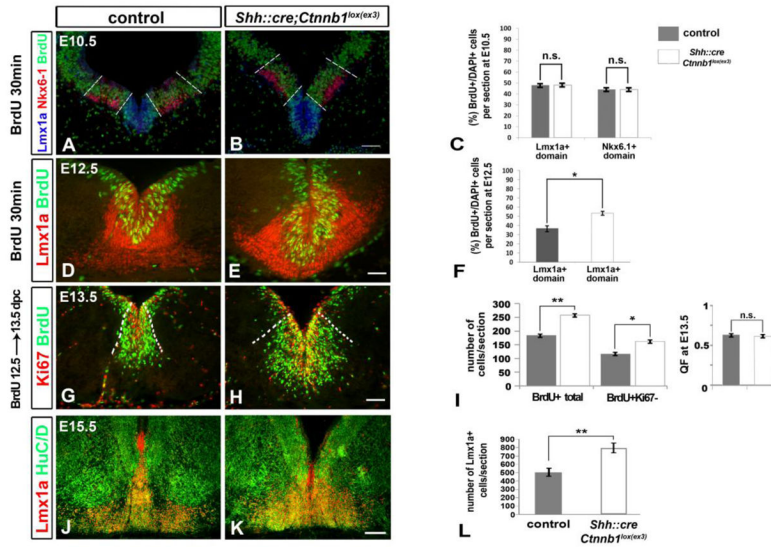


Figure 4. Excessive Wnt/beta-catenin signaling results in increased proliferation and number of mDA progenitors exiting the cell-cycle

(A–C) short pulse BrdU labeling at E10.5 reveals no change in total BrDU+ numbers (not shown) or the labeling index (BrdU+/DAPI+) of the *Lmx1a* and *Nkx6-1* domains between controls and mutants. (D–F) Short pulse BrdU labeling at E12.5. (D–F) An increase ($p < 0.05$) in the labeling index at E12.5 of *Shh::cre; Ctnnb1^{lox(ex3)}* mutants ($n=3$; $53.4\% \pm 1.2$ s.e.m) was observed when compared with controls ($n=3$; $37\% \pm 3.3$ s.e.m) within the *Lmx1a* + domain. A significant increase ($p < 0.001$) in the total number of BrdU+ cells per section was observed in mutants ($n=3$; 284 ± 28 s.e.m) when compared to controls ($n=3$; 135 ± 10 s.e.m). (G–I) BrdU was administered at E12.5 and embryos harvested at E13.5 to assess the number of mDA progenitors that have exited the cell-cycle between these two time points. (G and H) Sections were labeled with Ki67 and BrdU antibodies. Dotted lines indicate the dopamine field determined by *Lmx1a* labeling on the same or adjacent sections (not shown). In this *Lmx1a*+ dopaminergic field, the cells were counted and quantified in I. (I) A significant increase ($p < 0.01$) in the total number of BrdU+ cells per section was observed in mutants ($n=3$; 258.1 ± 8.3 s.e.m.) compared to controls ($n=3$; 184.4 ± 9.0 s.e.m.). In addition, an increase ($p < 0.05$) in the number of post-mitotic, BrdU+/Ki67– cells was detected in mutants ($n=3$; 161.6 ± 5.8 s.e.m) compared to controls ($n=3$; 119.5 ± 9.2 s.e.m). The quit fraction (BrdU+;Ki67-/BrdU+ total) was not significantly altered at this stage. (L–N) E15.5 coronal midbrain sections were labeled with *Lmx1a* and HuC/D antibodies in the control (L) and mutant (M). (N) A significant increase ($p < 0.01$) in the average number of *Lmx1a*+ cells per section is observed in the mutants ($n=3$; 789 ± 67 s.e.m.) in comparison to controls ($n=3$; 503 ± 51 s.e.m.). Scale bars: $50\mu\text{m}$ in A, B, D, and E; $100\mu\text{m}$ in G, H, J and K.

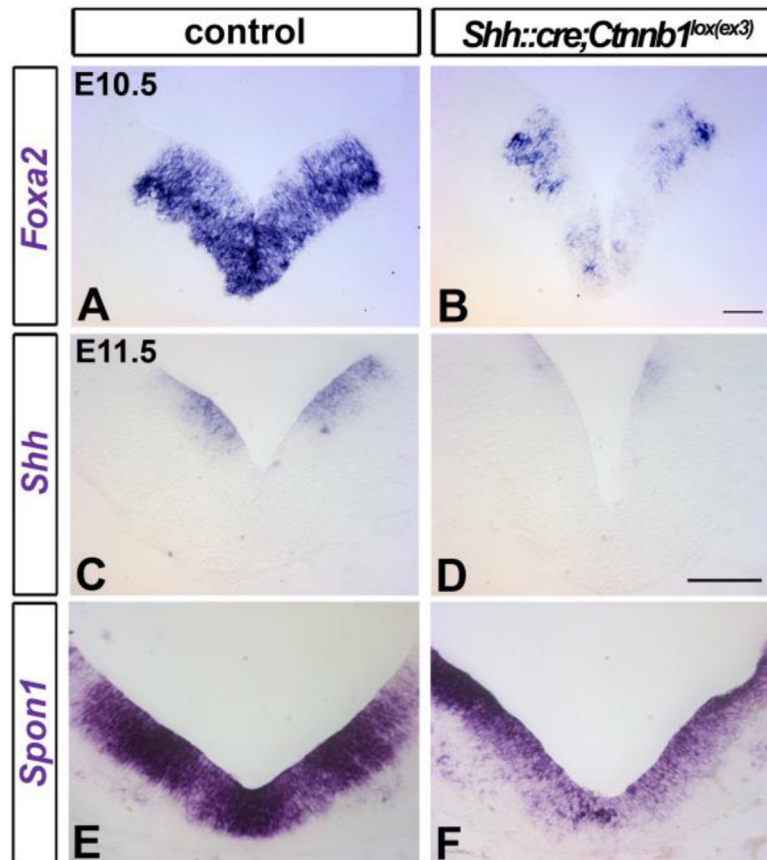


Figure 5. Excessive Wnt/beta-catenin signaling results in downregulation of floor plate genes E10.5 (A and B) and E11.5 (C–F) coronal midbrain sections were labeled with *Foxa2*, *Shh* and *Spon1* riboprobes in control (A, C, and E) and *Shh::cre;Ctnnb1^{lox(ex3)}* mutant embryos (B, D, and F). Expression of *Foxa2* (A and B), *Shh* (C and D), and *Spon1* (E and F) is reduced in the mutants. Note that in most mutants at this age, and more prominent at later stages, a deeper indentation at the ventral midline is observed, and the angle of ventrolateral midbrain is altered with respect to the midline. Scale bars: 50 μ m in A and B; 100 μ m in C–F.

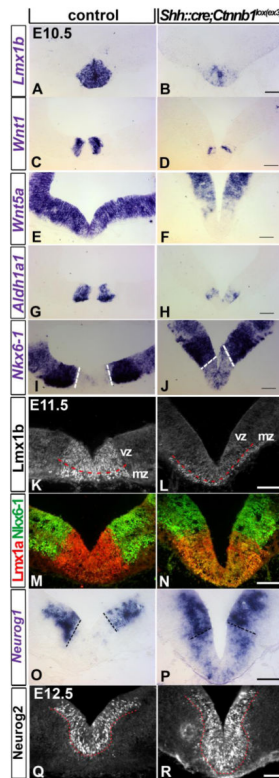


Figure 6. Excessive Wnt/beta-catenin signaling results in vacillating mDA progenitors
 E10.5 (A–J), E11.5 (K–P) and E12.5 (Q and R) coronal midbrain sections were labeled with indicated antibodies (K–N, Q and R) or *Lmx1b*, *Wnt1*, *Wnt5a*, *Aldh1a1*, *Nkx6-1* and *Neurog1* riboprobes in control (A, C, E, G, I, and O) and *Shh::cre;Ctnnb1^{lox(ex3)}* mutant embryos (B, D, F, H, J, and P). *Lmx1b*, *Wnt1*, *Wnt5a*, and *Aldh1a1* are all downregulated (B, D, F, and H; in the case of *Aldh1a1* n=5/7 mutants) while *Lmx1a* is minimally if at all reduced (N). Increased midline expression of *Nkx6-1* (J and N) and *Neurog1* (P) in mDA progenitors can be seen in the mutant embryos. In controls, we observed very low levels of *Nkx6-1* expression (I) and occasional *Neurog1*+ cells (O) at the midline. Red (K, L, Q, and R) dotted lines indicate a separation between the ventricular (vz) and mantle zones (mz). White (I and J) and black (O and P) dotted lines delineate the dopaminergic field. Scale bars: 50 μ m for A–J and 100 μ m for K–R.

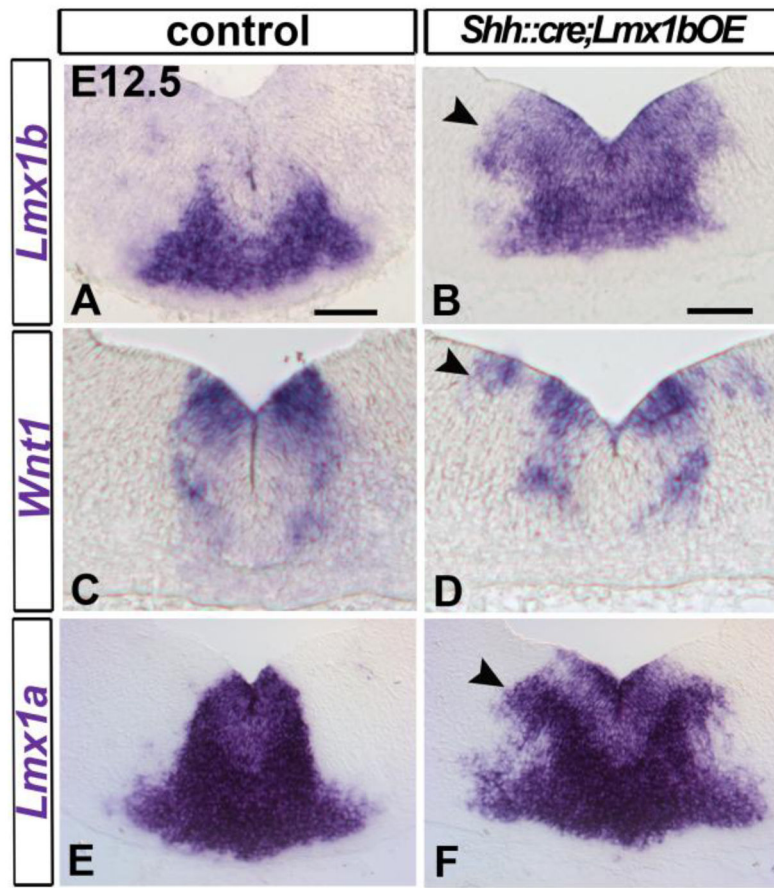


Figure 7. Maintenance of *Lmx1b* signaling alters ventral midbrain patterning
 E12.5 (A–F) coronal midbrain sections were labeled with *Lmx1b* (A and B), *Wnt1* (C and D), and *Lmx1a* (E and F) riboprobes in control and *Shh::Cre,Lmx1bOE* mutant embryos. *Lmx1b* is expanded dorsally in mutants (arrowhead in B) when compared to control. Similarly, *Wnt1* is increased in the ventricular zone (arrowhead in D) of the *ShhCre::Lmx1bOE* mutant. The *Lmx1a* expression boundary also expands dorsally as a consequence of increased *Lmx1b* expression in mutants (arrowhead in F) when compared with control. Scale bars: 50 μ m A–F.

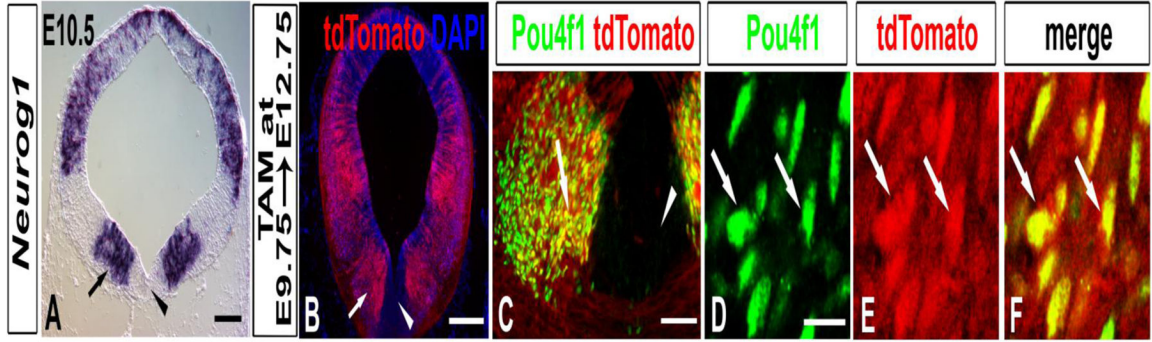


Figure 8. In normal embryos, *Neurog1*+ progenitors give rise to *Pou4f1*+ red nucleus neurons (A) E10.5 coronal midbrain sections were labeled with *Neurog1* riboprobe. Note that *Neurog1* is expressed in the progenitor region (arrow) immediately adjacent to the predominantly *Neurog1*- midline (arrowhead) that corresponds to the position of mDA progenitors. (B–F) Lineage of *Neurog1* progenitors in *Neurog1::creER^{T2}, Ai9* embryos. Tamoxifen was administered at E9.75 and embryos were harvested at E12.75. Consistent with the *Neurog1* expression pattern (A), tdTomato+ cells can be seen in the region (Arrows; B and C) just lateral to the largely negative medial region (Arrowheads; B and C) where mDA progenitors and neurons are situated. Several of these *Neurog1*+ descendants are *Pou4f1*+ red nucleus neurons (arrows; C–F). C is a higher magnification of the right side of the ventral midline roughly corresponding to the area indicated by the arrow and arrowhead in B. D–F panels show high magnification confocal images of the region corresponding to the area indicated by the arrow in C. Separate channels and the merged image are shown in D–F. The section in B is counterstained with DAPI to visualize nuclei. Scale bars: 100µm in A; 200µm in B; 50µm in C; 10µm in D–F.

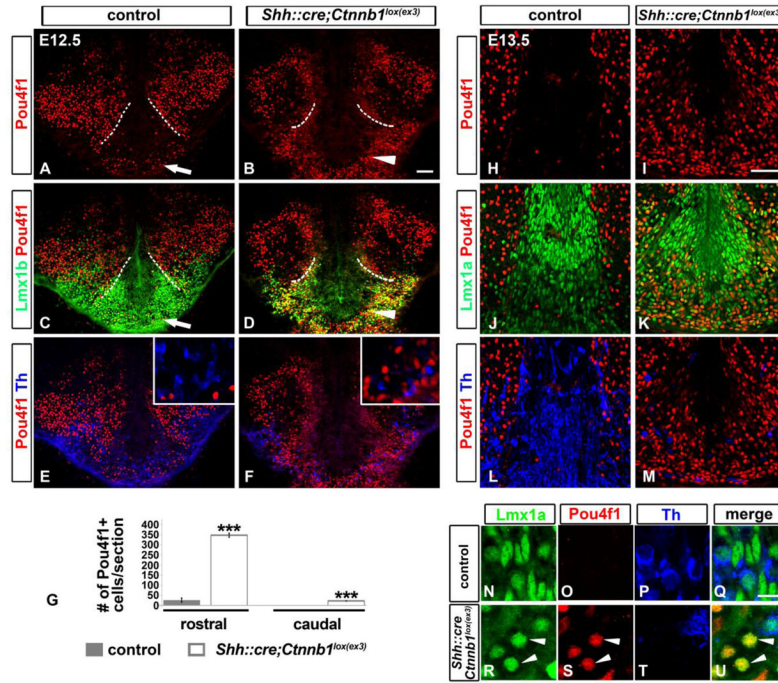


Figure 9. Excessive Wnt/beta-catenin signaling increases the number of Pou4f1+ neurons within the midbrain dopaminergic field

E12.5 (A–F) and E13.5 (H–U) coronal midbrain sections were labeled with indicated antibodies in control (A, C, E, H, J, L, and N–Q) and *Shh::cre;Ctnnb1^{lox(ex3)}* mutant embryos (B, D, F, I, K, M, and R–U). Note that Pou4f1+/Lmx1b+ cells appear to be in excess in the mutant (arrowheads in B and D) comparing to the control (arrows in A and C) within the dopaminergic field while Th+ neurons are reduced in the mutant. White dotted lines (A–D) delineate the dopaminergic field. Images were taken from the same section triple-labeled for Pou4f1, Lmx1b, and Th and are shown in a two-color combination. Insets in E and F are high magnification confocal images of the ventral midline in control and mutants, respectively. (G) A significant increase ($p < 0.001$) in a total number of Brn3a+ cells per section within the dopaminergic field is observed in the mutants ($n = 3$; rostral $347 \pm \text{s.e.m } 32$; caudal $24 \pm \text{s.e.m } 7$) in comparison to the control ($n = 4$; rostral $28 \pm \text{s.e.m } 10$; caudal $0.2 \pm \text{s.e.m } 0.1$). (H–M) Confocal images were taken from the same E13.5 section triple-labeled for Pou4f1, Lmx1a, and Th and shown in a two-color combination. At E13.5, increased numbers of Pou4f1+ cells persist at the ventral midline in the mutants (I). Note that these midline Pou4f1+ cells express Lmx1a (K) but not Th (M). (R–U) At the mutant midline, Pou4f1 (arrowheads in S) and Lmx1a (arrowheads in R) are expressed in the same cells (arrowheads in U), but do not express Th (blue). Scale bars: 50 μm in A–F and H–M; 10 μm in N–U.