Abstract

Wnt5a is a morphogen that activates the Wnt/planar cell polarity (PCP) pathway and serves multiple functions during development. PCP signaling controls the orientation of cells within an epithelial plane as well as convergent extension (CE) movements. Wnt5a was previously reported to promote differentiation of A9–10 dopaminergic (DA) precursors in vitro. However, the signaling mechanism in DA cells and the function of Wnt5a during midbrain development in vivo remains unclear. We hereby report that Wnt5a activated the GTPase Rac1 in DA cells and that Rac1 inhibitors blocked the Wnt5a-induced DA neuron differentiation of ventral midbrain (VM) precursor cultures, linking Wnt5a-induced differentiation with a known effector of Wnt/PCP signaling. In vivo, Wnt5a was expressed throughout the VM at embryonic day (E)9.5, and was restricted to the VM floor and basal plate by E11.5–E13.5. Analysis of Wnt5a+/− mice revealed a transient increase in progenitor proliferation at E11.5, and a precociously induced NR4A2+(Nurr1) precursor pool at E12.5. The excess NR4A2+ precursors remained undifferentiated until E14.5, when a transient 25% increase in DA neurons was detected. Wnt5a+/− mice also displayed a defect in (mid)brain morphogenesis, including an impairment in midbrain elongation and a rounded ventricular cavity. Interestingly, these alterations affected mostly cells in the DA lineage. The ventral Sonic hedgehog-expressing domain was broadened and flattened, a typical CE phenotype, and the domains occupied by Ngn2+ DA progenitors, NR4A2+ DA precursors and TH+ DA neurons were rostrocaudally reduced and laterally expanded. In summary, we hereby describe a Wnt5a regulation of Wnt/PCP signaling in the DA lineage and provide evidence for multiple functions of Wnt5a in the VM in vivo, including the regulation of VM morphogenesis, DA progenitor cell division, and differentiation of NR4A2+ DA precursors.

Introduction

Wnts comprise a family of 19 lipid-modified secreted glycoproteins that signal via different pathways and regulate multiple aspects of development [1,2]. These pathways include the canonical Wnt/B-catenin, noncanonical Wnt/Ca2+ and noncanonical Wnt/planar cell polarity (PCP) pathways.

Wnt5a has been reported to activate both canonical and noncanonical signaling depending on receptor, cellular and tissue context [3–5]. However, Wnt5a is generally considered a noncanonical Wnt that activates PCP or Ca2+ signaling [6]. Most PCP genes were initially identified in Drosophila or Xenopus and their homologues were subsequently found in mammals. These include genes for transmembrane proteins, such as fuziled (fit/Fz) [7–9], Von Gogh/Strabismus (Vang/Vangl/stbm) [10,11], and starry night/flammula/Celsa (stan/fin/Celsa) [12,13]. Some cytoplasmic components of this pathway are shared with the Wnt/B-catenin pathway, such as Dishevelled (dsh/Dvl) [14,15] and Casein kinase 1 (Ck1) [16–18]. Specific Wnt/PCP cytoplasmic components include Dna1, small GTPases of the Rho family: Cdc42, Rac1, Rhod, the Rho kinase and JNK [19–22].

Mutations in PCP genes produce specific and distinctive phenotypes. These include the general convergent extension (CE) defects seen in the overall shortened and broadened morphology of mutants and in Keller explants of the Xenopus dorsal marginal zone [23,24], the Drosophila wing with misdirected bristles and disorganization of the compound eye [2,25], the murine cochlea with misdirected hair cells [4,26], and the murine
Wnt5a and PCP/Neurogenesis

We have previously shown that Wnt5a promotes the differentiation of dopaminergic (DA) neurons in primary midbrain cultures and that Wnt5a signals via Dishevelled and Casein Kinase 1 in a dopaminergic cell line [17,33]. Wnt5a is known to activate the PCP pathway and to signal via small GTPases in different systems [34], but it is unknown which of the small GTPases transduces the Wnt5a signal in cells of the DA lineage. We therefore first investigated whether Wnt5a could activate Rac1, RhoA or Cdc42 in a DA cell line, SN4741, a validated model for studying Wnt signaling [33,35–37]. We found that treatment with recombinant mouse Wnt5a induced the activation of Rac1 (Figure 2A), while RhoA and Cdc42 activity were unchanged. In order to verify whether Rac1 mediates the pro-differentiation effects of Wnt5a, we used an in vitro assay in which Wnt5a induces the differentiation of primary DA precursors into DA neurons [32]. A dose-response curve for NSC 25766, a Rac inhibitor, showed that 10 μM had no effect on TH+ cell number. At higher doses, from 50 μM and up, cell death was seen (data not shown). Interestingly, treatment of these cultures with 10 μM of the Rac inhibitor NSC 25766 blocked the increase in the number of TH+ neurons otherwise induced by Wnt5a after 3 days in vitro (Figure 2B, C), suggesting that the pro-differentiation effects of Wnt5a are mediated by Rac1. Note that the morphology of the TH+ cells is unchanged, and that the cells appear healthy in all conditions (Figure 2C).

In the next part of our study we analyzed the VM phenotype of Wnt5a−/− mice generated previously [38]. We have previously reported that Wnt5a does not activate canonical signaling in a dopaminergic neuron cell line [33]. Data in the literature suggests that Wnt/β-catenin signaling could be decreased in Wnt5a−/− mice, since Wnt5a has been reported as capable of activating Wnt/β-catenin signaling [3], or increased since non-canonical signaling has also been reported to inhibit Wnt/β-catenin signaling [39]. To address possible regulation of canonical signaling in the Wnt5a−/− VM, we first examined the expression of Wnt1, a Wnt expressed in the VM that activates the Wnt/β-catenin pathway and is required for VM DA neuron development [40]. In situ hybridization did not show any increase in signal, but rather a wider spacing of the two ventral stripes of Wnt1 expression in the FP (Figure S1A). This was confirmed by QPCR analysis of E12.5 WT and Wnt5a−/− VMs tissue (Figure S1B), finding that argues against a significant imbalance between Wnt1 and Wnt5a. To examine canonical signaling at the effector level, we investigated the levels of active β-catenin by Western blot in the VM of E10.5 and E12.5 Wnt5a−/− mice, where no difference was detected (Figure S1C and data not shown). In sum, Wnt5a activated the small GTPase Rac1 and promoted DA differentiation via Rac1, but loss of Wnt5a had no effect on Wnt1 expression or canonical Wnt-signaling via β-catenin, suggesting that Wnt5a may regulate Wnt/PCP signaling in the ventral midbrain in vivo.

Differentiation of dopaminergic neurons is altered in Wnt5a−/− mice

To assess the role of Wnt5a in vivo, we examined the A9–A10 DA neuron populations in Wnt5a knockout mice at E11.5, E12.5, E14.5, E17.5 and E18.5. Surprisingly, at E11.5 and E12.5, the number of TH+ DA neurons in the Wnt5a−/− mice was not statistically different from wild-type (WT) littermate controls (Figure 3A, D). This was confirmed by quantitative PCR (QPCR) for TH and for Pitx3 (Figure 3 A,B), a homeobox transcription factor required for DA neuron survival [41–43]. A transient 25% increase in the number of TH+ DA neurons was detected at E14.5.
Figure 1. Temporal and spatial expression of Wnt5a in the mouse. (A) In situ hybridization for Wnt5a on coronal midbrain sections of E9.5–E13.5 CD1 mice shows a dynamic regulation of Wnt5a expression domains throughout development in both rostrocaudal distribution and developmental stages. Schemes with sagittal sections of the brain and dashed lines show the levels at which rostral (r) or caudal (c) expression analysis was performed. The expression of Wnt5a occupies the entire neuroepithelium of the ventral midbrain at E9.5 and 10.5, and becomes progressively restricted to the floor plate and basal plate ventricular zone from E11.5–E13.5, extending to the marginal zone at caudal levels (Scale bars at E9.5 = 100 μm, at E10.5 and E11.5 = 200 μm, at E12.5 and E13.5 = 175 μm). (B) At E12.5, the expression of Wnt5a comprises the FP extending from the ventricular zone through the intermediate zone and into the marginal zone, overlapping with NR4A2+ and TH+ cells. This extension into the marginal zone is most pronounced in the caudal midbrain. (C) The expression of Wnt5a is down-regulated by E18.5, where it still overlaps with TH+ cells, but 8 weeks after birth, at P56, this overlap is lost as seen in sagittal and coronal sections. (Scale bars in brightfield E18.5, coronal P56 and darkfield sagital P56 = 500 μm, scale bar in brightfield sagital P56 = 2.5 mm). Abbreviations: r = rostral, c = caudal, F = forebrain, D = diencephalon, M = midbrain, H = hindbrain.
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positive (BrdU+) cells [32]. To assess the role of Wnt5a in proliferation in vivo and the reason for the transient 25% increase in TH+ cells in the Wnt5a−/− embryos, the density of BrdU+ cells/area in the FP and the alar plate (AP) of E11.5 Wnt5a−/− and WT embryos were compared (black squares in Figure 4A). While the density of BrdU+ cells in the AP of WT and Wnt5a−/− embryos was not different (after a 2 hour pulse), a 35% increase in BrdU+ cells/area was detected in the FP of Wnt5a−/− mice (Figure 4A, B). Furthermore, BrdU+ cells accumulated in the ventral midline of the Wnt5a−/− midbrain at E11.5 (Figure 4A). The increase of proliferating cells within the FP was first evident at E11.5. This was confirmed by a 45% increase in Ki67+ cells (a marker of cells in the cell cycle) detected in Wnt5a−/− mice (Figure 4A, B). Similarly, the number of cells that are dividing (mitotic cells, arrowheads, Figure 4A) increased by 25% in Wnt5a−/− mice at E11.5 (Figure 4A, C). Interestingly, the increase of cells in S-phase and M-phase was maintained along the entire antero-posterior axis of the VM.

Neurogenesis in the VM is controlled by a code of homeodomain and proneural bHLH transcriptional regulators [44]. Expression of the members of the bHLH superfamily Ngn1, Ngn2 and Mash1 can be subdivided into three distinct domains within the VZ of the mouse VM [45]. Ngn2 is essential for proper DA neurogenesis, and Mash1 is required for the generation of VM GABAergic neurons [45–47]. The expression of Ngn2 (and the low levels of Mash1) in the midbrain FP therefore define the DA progenitor domain, whereas the high expression levels of Ngn2 and Ngn1 in the adjacent basal plate (BP) demarcate the oculomotor (OM) and red nucleus (RN) progenitor domains [45,46,48]. In the Wnt5a−/− embryos, the Ngn1-positive domain within the BP and the Ngn2-positive domain comprising the FP and BP were broadened in the mutant midbrain at E11.5 (Figure 5A), whereas the expression domains of these two proneural factors remained unchanged in the dorsal midbrain. We next examined whether the increase in cell division and broadened neurogenic domain had generated more postmitotic cells, and found a 47% increase in the number of NR4A2+ postmitotic cells compared to control at E12.5, suggesting that the neurogenic process is indeed enhanced in the Wnt5a−/− mice (Figure 5B, C). Furthermore, the 47% increase in NR4A2+ cells is consistent with a 45% increase in proliferating cells. However, a 25% excess of TH+ cells at E14.5 indicates that not all of the excess NR4A2+ cells differentiate into TH+ neurons at this point. Moreover, we found that the domain occupied by NR4A2+ cells was wider in Wnt5a−/− than in wild type mice (Figure 5B). An increase in the amount of NR4A2 mRNA was also detected at E12.5 by QPCR (Figure S2C).

We have previously reported that one of the functions of Wnt5a is to promote the differentiation of NR4A2+ DA precursors into DA neurons in vitro. This has been shown in diverse in vitro preparations, including rat E14.5 VM primary cultures [32], VM neurospheres derived from E10.5 mice or E12.5 rat [49] and mouse ES cells (unpublished observation). In order to investigate whether Wnt5a also promotes this differentiation step in vivo, we examined the proportion of TH+ cells out of NR4A2+ cells in the VM of Wnt5a−/− mice at E12.5. In agreement with our previous in vitro results, we found that the proportion of NR4A2+ cells differentiating into TH+ cells was reduced by 23% at this stage (Figure 5C). However, the number of TH+ cells increased at E14.5, and subsequently normalized, our results suggest that differentiation is rescued by an alternative mechanism. We thus conclude that Wnt5a is only partially and transiently required for the differentiation of NR4A2+ precursors into DA neurons in vivo.
Figure 3. The number of dopaminergic neurons is normal at most stages but transiently increases at E14.5 in Wnt5a<sup>−/−</sup> mice. (A) At E11.5 and E12.5 no differences in the number of TH<sup>+</sup> cells could be detected. At E12.5 and E14.5 the region occupied by TH<sup>+</sup> cells in the Wnt5a<sup>−/−</sup> midbrain appears larger, extending both laterally and dorsally. (B) At E17.5 the distribution of cells is broader dorsoventrally in rostral and caudal sections in the Wnt5a<sup>−/−</sup> VM. Importantly, at rostral levels the ventral tegmental area was more lateral (leaving a TH-poor midline domain) and the substantia nigra more medial, making it difficult to differentiate between them. (C) Enlarged image of TH<sup>+</sup> cells at E12.5 shows normal DA neuron morphology in the Wnt5a<sup>−/−</sup> mice. (D) Quantification of the number of TH<sup>+</sup> cells in E11.5, E12.5, E14.5, and E17.5 mice shows a transient 25% increase at E14.5 in Wnt5a<sup>−/−</sup> embryos, which was no longer seen at E17.5. (At E14.5, unpaired t-test, p = 0.0492, N = 4).

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Mediolateral/PCP and apico-basal morphogenetic defects in the VM of Wnt5a\(^{2/2}\) mice

Several of the defects described in previous sections are reminiscent of PCP developmental defects, including the accumulation of BrdU\(^+\) cells in the midline, the lateral expansion of the Ki67\(^+\), Ngn2\(^+\), NR4A2\(^+\), and TH\(^+\) domains in the VM as well as a flattening of the midbrain ventricle (Figures 3, 4, 5). Previous reports of animals lacking PCP components have shown that the invagination of the VZ in the ventral midline is flattened and that the Shh domain, that defines the FP and BP in the midbrain, is broadened [50–52]. In the Wnt5a\(^{2/2}\) mice, a lateral expansion of the Shh and Foxa2 expression domains was first detected at E11.5 (Figure 6A, Figure S2D,E). This was associated with a lateral expansion of the Lmx1a\(^+\) DA progenitor domain (Figure 6A). Expression of the Shh-target genes (Ptch1 and Gli1) was not expanded in the midbrain AP (Figure S3). The expression of class I (Dbx1) and class II (Nks2-2, Nks6-1) genes was not changed in the midbrain of the Wnt5a\(^{2/2}\) embryos compared to WT although the aberrant morphology of the Wnt5a\(^{2/2}\) midbrain noted previously led to a wider separation of the Nks6-1-positive domains in the BP of the mutant midbrain (Figure S3 and data not shown).

To characterize this defect in more detail, the analysis of the angle formed between the invagination of the ventricular epithelia and the midline in the VM revealed that this angle appeared greater in Wnt5a\(^{2/2}\) mice (59.4\(^\pm\)2.8\(^\circ\)), than in WT mice (49.1\(^\pm\)4.3\(^\circ\)) (Figure 6B). This resulted in the midbrain ventricle adopting a "U"-shape in the Wnt5a\(^{2/2}\) mice instead of the typical "V"-shape in controls (Figure 6A, B). A similar phenotype, including a broadened Shh domain, has been observed in the neural tube of the PCP mutants Scribble and Vangl2 [50–52].

It has previously been shown that cadherins regulate intercellular adhesion in neural progenitors and that a disruption of the complex formed with \(\alpha\)- and \(\beta\)-catenin leads to increased Shh signaling and proliferation [53]. We therefore examined the levels of N-cadherin in Wnt5a\(^{2/2}\) mice by Western blot and found a reduction at E9.5, prior to the expansion of the FP (Figure 6C).

Moreover, since apico-basal polarity depends on PCP and adhesion, we examined apico-basal polarity of cells in the ventricular zone. Interestingly, the change in the general morphology of the VM neuroepithelium was accompanied by an alteration in the orientation of the cells, as shown by the non-uniform orientation of propidium iodide stained nuclei in the Wnt5a\(^{2/2}\) mice, compared to WT at E12.5 (Figure 7A). To assess this quantitatively, the angle formed by the longest axis of each nuclei with the ventral midline was measured for 10 cells (starting from the midline and counting laterally) at different positions in the VM.
antero-posterior levels of the VM. This angle changed from very acute to less acute as the cells were positioned further away from the midline in the wild type. This angle reached almost 30° in the wild type, but did not exceed 30° degrees in Wnt5a−/− mice (Figure 7B). These results reflect the fact that, in Wnt5a−/− mice, the nuclei of apical FP cells are oriented ventrally, while the nuclei of control mice are oriented more ventro-laterally (Figure 7A, B). Furthermore, when the frequency of nuclei aberrantly oriented towards the contralateral side was examined, Wnt5a−/− mice showed a 7-fold increase compared to WT (Figure 7A, C). Note that while cells in the midline of both WT and Wnt5a−/− mice exhibited nuclei oriented contralaterally, only Wnt5a−/− mice showed nuclei with contralateral orientation in lateral positions of the FP (Figure 7A).

Several neuronal populations in the VM are redistributed following the PCP defect

A more detailed analysis of the distribution of A9–A10 DA neurons, in the very same animals that did not show any change in total DA cell number at E12.5, revealed changes in the mediolateral, rostro-caudal, and dorso-ventral axis (Figure 8A). Sections at regular intervals throughout the A9–A10 nucleus were examined. The three levels analyzed in WT and Wnt5a−/− mice (rostral, intermediate and caudal) are shown at E12.5 in (Figure 8A). In the mediolateral axis, TH+ cells extend more laterally in the Wnt5a−/− mice than in WT mice, especially in the rostral portion of the A9–A10 nuclei (Figure 8A–D). This phenotype persisted until E17.5, and followed the earlier morphological defects in the distribution of progenitor (Shh, Foxa2, Lmx1a, Ngn2) and precursor (NR4A2) markers, all of which were expanded already at E11.5.

In the rostro-caudal axis, the distribution of TH+ cells was unchanged at E11.5 (Figure 8E). However, at E12.5 and E14.5, the A9–A10 nuclei were shortened in Wnt5a−/− mice, seemingly at the expense of the most anterior portion of the nuclei (Figure 8F, G). Finally, by E17.5 the distribution of VM DA neurons in the antero-posterior axis tended to normalize but remained broad in the dorso-ventral axis (Figure 3B). This shortening of the anterior neural tube at E12.5 was also apparent when the length of the En1 expression domain was measured in sagittal sections through the VM of Wnt5a−/− mice, and compared to WT (390 μm compared to 540 μm, Figure 8J).

To distinguish between a deregulation of TH and a true misplacement of the DA neurons, we examined the spatio-temporal expression pattern of other DA neuron markers such as Pitx3 and Slc6a3/Dat. Both marker genes were expressed within the marginal zone (MZ) of the Wnt5a−/− ventral midbrain at E12.5 (Figure 8H).

A mediolateral expansion of the Th, Ptx3 and Slc6a3/Dat expression domains was detected in the Wnt5a−/− mice, confirming the redistribution of DA neurons within the VM (Figure 8F).

Since Wnt5a regulates PCP in several structures and the Shh, Foxa2 and Ngn1 domains were also broadened in the BP, we examined whether deletion of Wnt5a altered the distribution of other mature ventral neuronal populations in coronal midbrain sections at E18.5, when no difference in number of DA neurons was detected (data not shown). Interestingly, the area occupied by Th−, Isl1+ or Brn3a+ expressing cells was increased in Wnt5a−/− mutants (30–40%, Table 1). These results indicate that while the absolute numbers of midbrain neuron populations such as DA neurons are unchanged, the cells are re-distributed and positioned in a laterally and dorso-ventrally enlarged domain in the Wnt5a−/− VM (Table 1 and Figure 3).

Discussion

The impact of deletions of Wnt/PCP signaling components [2,29], including ligands such as Wnt5a [4], have been studied in very specific structures that have become standard Wnt/PCP functional assays. These include studying the orientation of bristles on the Drosophila wing, and in mammals the orientation of hair cells in the inner ear, convergent extension movements during embryo elongation, or neural tube closure. However, Wnt/PCP signaling components and ligands are expressed in very diverse tissues and at multiple developmental stages, where no previous PCP phenotypes have been described. In our study we investigated the function of Wnt5a in one such structure and developmental time, the VM during neurogenesis. We report that Wnt5a regulates VM morphogenesis, limits DA progenitor proliferation and enhances DA precursor differentiation.

Regulation of DA precursor differentiation by Wnt5a

Despite the clear effects of Wnt5a on DA differentiation in both gain and loss of function experiments in vitro [32,54], Wnt5a−/− mice exhibited only a mild and transient DA differentiation phenotype in vivo. Moreover, in addition to a decrease in the proportion of NR4A2+ precursors differentiating into TH+ DA neurons (25% decrease in the Wnt5a mutants compared to WT),
we found that NR4A2+ DA precursors were generated in excess by E12.5 (47% in the Wnt5a mutants compared to 39% in WT). These results suggest that both progenitor proliferation and the differentiation of NR4A2+ precursors were affected in the Wnt5a mutants. These alterations were transient and were not detected at E14.5, when the number of TH+ cells increased by 25%, then returned to control level at E17.5 and E18.5. Thus, our results show that Wnt5a is only transiently required for the differentiation of endogenous midbrain NR4A2+ precursors in vivo, and is sufficient for their differentiation in vitro, via Rac1 activation. Interestingly, the surprising alteration in the kinetics of DA neurogenesis in the Wnt5a-/- mice suggests that other phenomena such as increased neurogenesis (increased number of DA postmitotic precursors) and a compensatory mechanism at E14.5, may prevent a stronger DA differentiation phenotype. Indeed, several non-canonical Wnts are expressed in the VM [37], and a functional redundancy between wnt5 and wnt11 has been described in double zebrafish ppetal (ppt)/wnt5 and silberblick (shb)/wnt11 mutants [50], suggesting that another non-canonical Wnt may compensate for the loss of Wnt5a.

A role for Wnt5a in midbrain morphogenesis
The involvement of non-canonical Wnt-signaling in CE movements and neural tube morphogenesis has been widely documented [29,55,56]. Moreover, Wnt5a has recently been clearly implicated in the regulation of CE movements and PCP during cochlear development and neurulation in the mouse [4]. Interestingly, the mesencephalon undergoes unique morphogenic movements to form the cephalic flexure proper, a process that is coupled to CE movements in the ventral domain and outgrowth in the dorsal domain of the mesencephalon [57]. Moreover, Wnt5a is expressed exclusively in the VM and not dorsal midbrain throughout mouse embryonic development. Our analysis of the Wnt5a-/- mice has revealed a large variety of subtle but clear alterations in the morphogenesis and the cytoarchitecture of the VM. Overall the VM was broader and shorter as demonstrated by: a lateral expansion of Shh-expressing FP/BP at E11.5, accompanied by broader lateral expression of Foxa2, Lmx1a, NR4A2, Th, Pitx3 and Dat. Other markers examined were also more laterally placed such as Wnt1 and Nkx6.1. The shortened midbrain phenotype was manifested in the number of levels with TH+ cells and their distribution, and suggested by a reduced length of En1 expression in sagittal sections. In coronal sections, the mesencephalon did not acquire its distinct heartlike morphology and the ventricle in the ventral midline adopted a wide “U” shape instead of the typical “V” shape at E10.5. Thus, our findings suggest diminished CE and an involvement of Wnt/PCP signaling in the VM, as described for other structures [4,22,24,28,58].
Interestingly, we also found a change in the apico-basal orientation of midbrain VZ cells in Wnt5a−/− mice compared to WT. The axis of individual WT FP apical ventricular cells and the midline formed an average 36° angle and did not cross the midline ventrally. However, cells in the Wnt5a−/− mice formed an average 20° angle with the midline and in some instances individual cells showed a negative angle (i.e. their axis pointed contralaterally). A possible interpretation of these results is that the increase in proliferation allows nuclei to be oriented aberrantly in Wnt5a−/− mice during mitosis. However, misorientation was never seen in WT mice in lateral positions, regardless of cell cycle phase, but was seen in Wnt5a−/− mice. This could also reflect a consequence of the flattened VM morphology, allowing greater deviations towards the contralateral side. A more intriguing possibility is that the alteration in PCP directly regulates or causes secondary changes in the geometry of the cells and in apicobasal polarity. PCP and apicobasal polarity require cell attachment, a process that both pathways also regulate [59,60]. In line with this, we found a decrease in the levels of N-cadherin in the Wnt5a−/− mice at E9.5, which could contribute to decreased attachment and altered polarity. These results suggest PCP and apico-basal polarity may be coordinately regulated in certain structures by adhesion proteins.

Triple slkppmut-morphant zebrafish embryos phenocopy the neurulation defect of trilobite (tri)/Van Gogh-like 2 (Vangl2) PCP mutants [61]. Interestingly, the defects in the Wnt5a−/− VM resemble the tri neurulation defects with an increase in proliferation, a broadening of the Shh domain/expansion of the FP, and an ectopic accumulation of neural progenitors within the midline [61]. These findings strongly suggest that Wnt5a also regulates cell intercalation after cell division and VM morphogenesis. Our analysis of the VM phenotype of Wnt5a−/− mice suggests a model in which Wnt5a regulates morphogenesis by a mechanism involving cell adhesion and altered PCP signaling. This is accompanied by an alteration in apico-basal polarity, and a broader distribution of VM cell populations, but no alteration in the final number of neurons, all of which is compatible with a PCP phenotype.

**Proliferation: a link between differentiation and morphogenesis**

Our results indicate that the loss of Wnt5a directly or indirectly regulates cell division in VZ progenitors. Indeed, we found that deletion of Wnt5a increased the number of both BrdU+ and Ki67+ cells in the apical VZ, and the number of NR4A2+ postmitotic cells in the IZ, which suggested a regulation of both proliferative and neurogenic divisions. Interestingly, Wnt/PCP signaling has been previously reported to regulate oriented cell division during gastrulation, allowing axis elongation [61,62], and to regulate asymmetric cell division [63], which is the predominant mode of division used by VM progenitors during DA neurogenesis. Another mechanism that could also result in the regulation of proliferation and neurogenesis is cell adhesion. Our results indicate that Wnt5a may regulate cell adhesion in the neuroepithelia by regulating the levels of N-cadherin as early as E9.5. Interestingly, it has been described that a reduction in α-catenin

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**Figure 7. Apical-basal polarity/cell orientation are affected in Wnt5a−/− mice.** (A) At E12.5, propidium iodide staining on coronal sections through the VM revealed that cell nuclei in the neuroepithelium were rounded and their orientation was more variable with some cells pointing contralaterally (red asterisks/arrows). (B) The orientation of each cell nucleus was plotted versus its distance from the ventral midline. The angle between the nucleus and the midline was measured from cell 1 (the most medial) to cell 10 (the most lateral). Cell nuclei in Wnt5a−/− mice are oriented more ventrally compared to the more lateral orientation of cells in WT mice (two way-ANOVA for genotype and level, p = 0.0029, N = 3). (C) The frequency of cell nuclei oriented towards the contralateral ventral side (red arrows in A) was significantly increased in Wnt5a−/− mice (paired t-test, p = 0.0198, N = 3, 10 nuclei at 3 levels/animal).

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binding to N-cadherin leads to decreased cell adhesion, increased Shh signaling and proliferation [53]. We therefore suggest that the increase in Shh signaling (first detected at E11.5 by the regulation of Foxa2 and Lmx1a), may be induced by a decrease in the levels of N-cadherin protein and cell adhesion at earlier stages, when no other phenotype is apparent. In agreement with a role for Shh in regulating proliferation, we found an increase in the number of proliferating apical VZ progenitors and a lateral expansion of the...
Shh, Foxa2 and Lmx1a domains in the Wnt5a−/− mice. Thus, taken together, these data suggest that Wnt5a can affect both the extent and the type of cell division in VZ progenitor cells by different mechanisms.

In sum, our results show that CE defects and lowered cadherin expression were the first detected phenotype at E9.5 in the Wnt5a−/− mice. This was followed by an alteration in morphogenesis and DA differentiation, and at E12.5 by defects in cell polarization and adhesion, a shortening and a broadening of the A9-A10 nucleus and other ventral domains of the mesencephalon. We suggest that the broadening of the Shh domain may lead to an increase in Shh-signaling, increased proliferation in the FP/BP, accumulation of NR4A2 domain and a transient expansion of the Th+ nucleus and other ventral domains of the mesencephalon. We further suggest that Wnt5a regulates CE movements required for axial elongation, polar growth and morphogenesis as well as proliferation, neurogenesis, some aspects of differentiation, and the actual positioning of neurons in the VM.

Materials and Methods

Animals, Immunohistochemistry and in situ Hybridization (ISH)

Wnt5a+1/Lmcre [referred to as Wnt5a−/− in this article] transgenic mice [38] and CD1 mice (Charles River) were housed, bred and treated in accordance with the approval of the local ethics committee (Stockholms Norra Djurförsöketiska Namnd). Wnt5a+/− mice were kept on a C57BL/6 background. In all experiments, the Wnt5a+/− embryos were compared to their wild-type (Wnt5a+/+) and Wnt5a+/−, heterozygotes with wild-type phenotype) littermates, n=4 for each genotype, if not otherwise stated in the text. Mice of the relevant genotype were mated overnight and the type of cell division in VZ progenitor cells by proliferation in the FP/BP, accumulation of NR4A2 domain may lead to an increase in Shh-signaling, increased morphogenesis and DA differentiation, and at E12.5 by defects in cell polarization and adhesion, a shortening and a broadening of the A9-A10 nucleus and other ventral domains of the mesencephalon. We suggest that the broadening of the Shh domain may lead to an increase in Shh-signaling, increased proliferation in the FP/BP, accumulation of NR4A2 precursors and a transient expansion of the Th+ cell population at E14.5. These defects were most pronounced in the FP, thus affecting the morphology and development of cells in the DA lineage in the Wnt5a−/− mice.

In conclusion, we show for the first time that, in the VM, Wnt5a regulates CE movements required for axial elongation, polar growth and morphogenesis as well as proliferation, neurogenesis, some aspects of differentiation, and the actual positioning of neurons in the VM.

Radioactive in situ hybridization

Paraffin sections (8 μm) of mouse embryos (E11.5/E12.5) or brains (E18.5, P56) were processed for radioactive in situ hybridization as previously described [65]. The probes used were as follows: Wnt5a [38], Shh, Ebf1, Wnt1 [66], Th, Pax3, Scl6a2/Dat [65], Foxa2 (bp 743–1314, Acc. Nr. NM_010446), Dbx1, Isl1, Pou4f1, Nkx2-2, Nkx6-1 [67], Ngn1, Ngn2 [68], Lmx1a (bp 412–1211, Acc. Nr. NM_033652) Ptkh1 [69] and Gli1 [70]. Images were taken using darkfield optics on a stereo microscope Steini SV6, AxiosCam MRc camera and Axiosvision 4.6 software (Zeiss, Jena/Germany).

BrdU treatments

Pregnant females were injected intra-peritoneally with 5-bromo-2-deoxyuridine (BrdU, 10 μg/g body weight) two hours before they were sacrificed and processed as described [71]. The number of BrdU-positive cells in a square area (2500 μm2) was counted on at least four serial sections from the midbrain of stage-matched littermates (n=4) using the Neurolucida 6 software (MBF Bioscience, Williston, VT/USA).

TH+ Cell Counts and Distribution

For counts of total number or distribution of TH+ cells in the ventral midbrain, alternate 14 μm sections from coronal series of the entire A9-A10 population were counted and plotted versus position at E11.5 and E12.5 for distribution analysis. At E14.5 and E17.5, every sixth section was counted and plotted in the distribution analysis. The lateral spread of TH+ cells was measured in coronal sections through the VM, in three consecutive levels, where level 1 corresponds to the rostral midbrain-Pl boundary, level 2 corresponds to the intermediate midbrain and level 3 corresponds to the caudal midbrain (see Figure S 5). Pictures were taken with identical acquisition settings and magnification and the lateral spread was measured by drawing a horizontal line between the outermost TH+ cells. This distance was measured in pixels using ImageJ and then normalized to littermate controls.

The volumes of the TH−, Bm3a− and Isl1−expression domains at E18.5 were measured by the Cavalieri method using the Stereo Investigator 5.05.4 software (MBF Bioscience) as described [65]. Cell numbers were averaged for each genotype and subjected to a Student’s t-test for the estimation of statistical significance.

Table 1. The area occupied by the Isl1+, Brn3a+ or TH+ domain in coronal sections is 30–40% greater in Wnt5a−/− mice at E18.5.

| Genotype | Isl1+ domain (μm²×10⁴) n=3 | Brn3a+ domain (μm²×10⁴) n=3 | TH+ domain (μm²×10⁴) n=3 |
|----------|-----------------------------|-----------------------------|-----------------------------|
| Wildtype | 62.4±9.79 s.e.m. | 347.2±20.8 s.e.m. | 893.2±40.5 s.e.m. |
| Wnt5a−/− | 893.2±40.5 s.e.m. | 443.2±18.4 s.e.m. | 1140.2±93.4 s.e.m. |

The more ventral TH+ population shows the greatest difference.

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Calculation of slope of ventricular invagination and analysis of nuclei orientation

E10.5 and E12.5 mice were stained with Hoechst or propidium iodide respectively. The slope of the wall of the VM at E10.5 was measured by drawing a cross bisecting the FP, where the origin coincided with the ventricular invagination. A tangent to the ventricular wall was drawn and the angle between the tangent and the midline was measured in 4 embryos of each genotype. To measure the nucleus orientation, confocal pictures of serial sections of E12.5 midbrains were taken with an optical slice of 1 μm. Ten nuclei along the ventricle were analyzed per image from the midline outwards, 3 images per mouse (rostro-caudally distributed), and 3 mice per genotype were analyzed. The nucleus was outlined in ImageJ and the angle of the longest axis was measured compared to the vertical parallel to the midline. This gave an angle that was plotted against its position from the midline (nucleus number position). Certain nuclei were abnormally oriented towards the contralateral side, these gave a negative angle. The number of nuclei with abnormal orientation (negative angle), was compared to the total number of cells and this ratio was used for analysis of contralateral orientation.

Primary ventral mesencephalic cultures and immunocytochemistry

Ventral midbrains of E11.5 CD1 mice were dissected out in ice-cold PBS supplemented with 0.2%glucose, mechanically dissociated in serum-free N2 through flame-narrowed Pasteur pipettes and plated at a final density of 125,000 cells/well in poly-D-lysine-coated 48-well plates as described previously [32]. Cultures were treated with recombinant mouse Wnt5a (200 ng/ml, RnD), 0.05% CHAPS (Wnt5a control, Sigma), and/or 10 μM Rac1 inhibitor (NSC 23766, Calbiochem). Treatment of cultures was initiated 1 hour after plating and cultures were incubated for 3 days in N2 at 37°C in 5%CO₂. Cells were then fixed for 15 minutes with 4% paraformaldehyde, washed in PBS, and used for immunocytochemical analysis.

Real-time Quantitative PCR

cDNA was generated as described previously [32]. In brief, RNA from E8.5-P0 VMs of CD1 (for developmental stages) or wildtype and Wnt5a−/− mice was extracted using RNeasy Mini Kit (Qiagen). 1 μg of RNA was reverse-transcribed using SuperScript II Reverse Transcriptase (Invitrogen) and random primers (Invitrogen). Quantitative PCR and the primers used in this study have been described previously [32] with the exception of NR4A2 primers; Forward: 5'CAGCTCCGATTTCTTA' and Reverse: 5'GGTGAGGGTCCATGC-TAAACCGA3'.

Activated GTPase assay

Immunoblotting and sample preparation were performed as described [17]. SN4741 cells were cultured as described previously [17]. Cells were serum starved overnight and stimulated with recombinant Wnt5a (200 ng/ml, R&D) for 2 hours. Cells were washed with ice-cold PBS and lysed in GTPase lysis buffer (10 mM Tris-HCl, pH=7.5; 110 mM NaCl; 1 mM EDTA; 10 mM MgCl₂; /well, 1% Triton – x100; 0.1% SDS, 1 mM DTT, protease inhibitor cocktail (Roche)) for 5 minutes at 4°C. Cell lysates were cleared by centrifugation at 14 000 rpm/4°C/5 min and incubated for 15 min/4°C on a rotator with 25 μl of GST-PK-CRIB beads (25% slurry) for Rac1-gtp pulldown, GST-Rhotekin for RhoA-gtp pulldown and GST-WASP for cdc42-gtp pulldown. Beads were subsequently washed 3 times with 0.5 ml GTPase lysis buffer (without 0.1% SDS), mixed with 2× Laemmli buffer and analyzed on SDS-PAGE for the amount of activated RhoA/Rac1/cdc42. 5% of total cell lysate was used as input control.

Statistics

Data were analysed using Graphpad Prism version 4.01 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. For analyses comparing WT and Wnt5a−/− mice, t-tests were used. A paired t-test was used if 1WT/Wnt5a littermate pair was used from individual litters. If several pairs were taken from the same litter then an unpaired t-test was used. At least 3 litters were used for each analysis. Each graph value is the mean±SEM. For analyses of distribution of Rac inhibition of dopaminergic neuron differentiation and for dopaminergic neuron distribution in vivo, One-way and Two-way ANOVA were used, with Bonferroni’s post-hoc test.

Supporting Information

Figure S1 Canonical Wnt signaling is unaffected by loss of Wnt5a. (A) In situ hybridization at E12.5 for Wnt1 shows wider spacing between the two ventral stripes of Wnt1 expression in the Wnt5a−/− mice (red brackets), but no enlargement of the Wnt1 domain (Scale bar is 500 μm). (B) QPCR for Wnt shows no difference in levels of Wnt1 in the VM of Wnt5a−/− mice at E12.5. (C) Western blot for active (dephosphorylated) β-catenin shows little difference at E12.5 in the Wnt5a−/− VM. Found at: doi:10.1371/journal.pone.0003517.s002 (0.71 MB TIF)

Figure S2 QPCR of E12.5 WT and Wnt5a−/− VM. No difference in Th (A) and Ptx3 (B) mRNA levels. Increase in the amount of Nr4a2 mRNA (C). Small or no increase in Shh mRNA (D) and a greater difference in Foxa2 levels (E). Found at: doi:10.1371/journal.pone.0003517.s003 (5.40 MB TIF)

Figure S3 No change in dorsoventral patterning of the Wnt5a−/− midbrain. Expression of Sbh-target genes Ptkh1 and Gli1 was not altered in Wnt5a−/− mice. The expression of class I (Dbx1) and class II (Nkx6-1) genes was not changed in the midbrain of the Wnt5a−/− embryos compared to WT. Found at: doi:10.1371/journal.pone.0003517.s004 (5.40 MB TIF)

Figure S4 Anteroposterior levels used to analyze the lateral distribution of TH+ cells. Levels 1, 2 and 3 corresponding to rostral, intermediate and caudal levels are depicted on sagittal sections of WT and Wnt5a−/− mice, probed for Th. Found at: doi:10.1371/journal.pone.0003517.s005 (7.93 MB TIF)

Figure S5 The E11-expressing domain is shortened in Wnt5a−/− VM (A) Bright field and dark field images of the midbrain cephalic flexure hybridized for Engrailed1 (En1) show a shorter anteroposterior extension of E11 in the Wnt5a−/− midbrain. Red line shows the length measured from isthmus to anterior-most E11 expression. (B) Quantification of E11 expression length shows a shorter domain in Wnt5a−/− embryos [paired t-test p = 0.0203, WT N = 3, Wnt5a−/− N = 2].

Found at: doi:10.1371/journal.pone.0003517.s006 (7.4 MB TIF)

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Author Contributions
Conceived and designed the experiments: ERA NP LC VB ACH WW. Performed the experiments: ERA NP LC EM VB LB ACH. Analyzed the data: ERA NP LC VB LB ACH WW. Contributed reagents/materials/analysis tools: TPP WW. Wrote the paper: ERA NP WW.

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