Wnt2 Regulates Progenitor Proliferation in the Developing Ventral Midbrain*

Kyle M. Sousa†‡, J. Carlos Villaescusa†, Lukas Cajanek§, Jennifer K. Ondr‡, Goncalo Castelo-Branco†‡, Wytse Hofstra‡, Vitezslav Bryja¶, Carina Palmberg**, Tomas Bergman**, Brandon Wainwright‡‡, Richard A. Lang†, and Ernest Arenas‡³

From the 1Laboratory of Molecular Neuroendocrinology, Department of Medical Biochemistry and Biophysics, Karolinska Institute, Scheeles väg 1, A1:2, 17177 Stockholm, Sweden, the 2Developmental Biology Division and Department of Ophthalmology, Children’s Hospital Research Foundation, Cincinnati, Ohio 45229-3039, the 3Institute of Experimental Biology, Faculty of Science, Masaryk University & Institute of Biophysics AS CR, Kotlarska 2, 61107 Brno, Czech Republic, the 4°Department of Medical Biochemistry and Biophysics, Division of Chemistry I, Karolinska Institute, Stockholm SE-17177, Sweden, the 5Department of Molecular and Integrative Physiology, University of Michigan Medical School, Ann Arbor, Michigan 48109, and the 6¶Institute for Molecular Bioscience, The University of Queensland, St. Lucia 4072, Australia

Wnts are secreted, lipiddated proteins that regulate multiple aspects of brain development, including dopaminergic neuron development. In this study, we perform the first purification and signaling analysis of Wnt2 and define the function of Wnt2 in ventral midbrain precursor cultures, as well as in Wnt2-null mice in vivo. We found that purified Wnt2 induces the phosphorylation of both Lrp5/6 and Dvl-2/3, and activates β-catenin in SN4741 dopaminergic cells. Moreover, purified Wnt2 increases progenitor proliferation, and the number of dopaminergic neurons in ventral midbrain precursor cultures. In agreement with these findings, analysis of the ventral midbrain of developing Wnt2-null mice revealed a decrease in progenitor proliferation and neurogenesis that lead to a decrease in the number of postmitotic precursors and dopaminergic neurons. Collectively, our observations identify Wnt2 as a novel regulator of dopaminergic progenitors and dopaminergic neuron development.

Wnt ligands represent a large family of secreted intercellular signaling molecules important for vertebrate development (1). Wnts have been implicated in a variety of biological processes including proliferation (2), dorsal-ventral patterning (3), dendrite arborization (4), differentiation (5), and cell polarity (6, 7). Wnt-mediated signals can be transduced by Wnt/β-catenin (also called the canonical pathway) or the diverse non-canonical pathways that include both Wnt/Ca²⁺ and planar cell polarity signaling. Canonical Wnt signals are transduced via β-catenin, a key downstream effector. In this pathway, a Wnt ligand binds its cognate seven transmembrane receptor, Frizzled, and LRPS/6 coreceptor, phosphorylating the cytoplasmic mediator protein, Dishevelled (Dvl). Consequently, activation of Dvl results in the inhibition of glycogen synthase kinase-3β (GSK-3β), the stabilization (dephosphorylation) and translocation of β-catenin to the nucleus, and the activation of TCF/LEF target genes (8). Stimulation of non-canonical Wnt/Ca²⁺ signaling has been shown to trigger phospholipase C signals, increase intracellular calcium concentrations, and activate the downstream effectors protein kinase C, calcineurin, and Ca²⁺/calmodulin-dependent kinases (9, 10). In the planar cell polarity (PCP) pathway, Wnts activate Dvl to regulate cytoskeletal reorganization and cell adhesion via Rh-associated kinase (11–13).

To date, 19 different Wnts have been identified in the mouse genome that elicit a diverse set of cellular responses in many biological systems. Studies describing the analysis of Wnt1-null mice documented an important role for this gene in midbrain/hindbrain and dopaminergic (DA) neuron development (14–18). Accordingly, transgenic mice engineered to express β-galactosidase (TOPGAL) under the control of a TCF/LEF-inducible promoter exhibit TOPGAL activity in DA precursors of the developing ventral midbrain (19). Interestingly however, the ventral midbrain expresses several different Wnts, but their function is largely unknown (20). In vitro studies have provided evidence that different Wnts, including Wnt1, Wnt3a, and Wnt5a, have distinct activities in the developing midbrain (19, 21) and that the differentiation of DA precursors can be enhanced by either Wnt5a or stabilization of β-catenin with GSK-3β inhibitors (22). Analysis of null mutant mice has revealed that whereas Wnt1 is required for several aspects of DA neuron development such as specification, proliferation, neurogenesis, and survival (16–18), Wnt5a is required for VM morphogenesis and DA precursor differentiation (6). These findings highlight the importance of diverse Wnt signals for developing DA neurons and their pos-

* This work was supported in part by the Swedish Foundation for Strategic Research (INGVAR and CEDB), Swedish Research Council (VR2008:2811 and DBRM), Norwegian Research Council, European Commission (EuroStemCell), and Karolinska Institute.

1 Supported by a FEBS Long-Term fellowship.
2 Supported by the Portuguese Fundação para a Ciência e Tecnologia, Karolinska Institute, and the Calouste Gulbenkian Foundation. Present address: The Wellcome Trust/Cruk, Gurdon Inst., University of Cambridge, Cambridge CB2 1QH, UK.
3 To whom correspondence should be addressed: Dept. of Medical Biochemistry and Biophysics, Karolinska Institute, 17177 Stockholm, Sweden. Tel.: 46-8-728-7663; Fax: 46-8-341960; E-mail: ernest.arenas@ki.se.
4 The abbreviations used are: Dvl, Dishevelled; Nurr1, nurr77-related receptor; PD, Parkinson disease; DA, dopaminergic; TH, tyrosine hydroxylase; VM, ventral mesencephalon; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PBS, phosphate-buffered saline; HA, hemagglutinin; PFA, paraformaldehyde; ISH, in situ hybridization.
Purified Wnt2 Regulates Proliferation in the Midbrain

Purification of HA-tagged Wnt2—Purification of C-terminally tagged HA-Wnt2 was performed as previously described with minor modifications (31). Briefly, four liters of conditioned medium from rat B1A fibroblasts stably transfected with HA-Wnt2 (32) was collected and filtered through 0.45-μm filters. Next, CHAPS detergent was added to a final concentration of 1%, and the medium was subsequently filtered through a 0.2-μm filter prior to loading on Blue Sepharose 6 Fast Flow column (Amersham Biosciences). The Blue Sepharose column had been washed and equilibrated with two bed volumes of wash buffer containing 20 mM Tris, 150 mM KCl, pH 7.5, and one bed volume of 20 mM Tris, 150 mM KCl, 1% CHAPS, pH 7.5 (washing buffer). The column was then loaded with the HA-Wnt2-containing medium and washed extensively in washing buffer. Fractions were eluted using a high salt buffer (20 mM Tris, 1.5 M KCl, 1% CHAPS, pH 7.5) and analyzed by immunoblotting for the presence of HA tag (mouse anti-HA, Covance). HA-Wnt2-positive fractions were pooled and concentrated in Centricon Plus-80 filters for loading onto a HiLoad 26/60 Superdex 200 gel filtration column (Amersham Biosciences) using phosphate-buffered saline (PBS), 1% CHAPS, pH 7.5. Eluted fractions were analyzed further for the presence of HA-Wnt2 by immunoblotting and subjected to a final purification step using a HiTrap Heparin HP column using PBS, 1% CHAPS, pH 7.5 as loading buffer and PBS, 1 M NaCl, 1% CHAPS, pH 7.5 as elution buffer. The presence of HA-Wnt2 in eluted fractions was analyzed by immunoblotting using mouse anti-HA, (Covance) and anti-Wnt2 antibody (Abcam; ab277794). Silver staining was performed as previously described (21).

Ventral Precursor Cultures and Immunocytochemistry—Pregnant CD1 mice were sacrificed, and embryos were collected on embryonic day (E) 10.5, and primary ventral mesencephala were dissected, mechanically dissociated, and plated at a density of 125,000 cells/well in poly-d-lysine-coated 48-well plates. Cells were treated with 40 ng of purified HA-Wnt2 and grown for 3 days in vitro in defined serum-free N2 media consisting of a 1:1 mixture of F12 and DMEM supplemented with insulin (5 mg/ml), apo-transferrin (100 mg/ml), putrescine (100 μM), progesterone (20 nM), selenium (30 nM), glucose (6 mg/ml), and bovine serum albumin (1 mg/ml). Cells were fixed with cold 4% paraformaldehyde (PFA) for 15 min prior to immunocytochemical analysis. Cultures were blocked for 1 h at room temperature in PBST (PBS, 1% bovine serum albumin, and 0.3% Triton X-100) and overnight at 4 °C with the corresponding primary antibody diluted in PBST. The following antibodies were used: mouse anti-β-tubulin type III (TuJ1), 1:2000 (Sigma); rabbit anti-TH, 1:250 (Pel-freeze). After washing, cultures were incubated with secondary antibodies for 2 h using 1:100 dilutions of Cy2- or rhodamine-coupled secondary antibodies (Jackson ImmunoResearch). Cultures were then rinsed twice in PBS and analyzed using a Zeiss Axiosvert 100M microscope, and images were taken with a Hamamatsu C4742–9 camera with QED CAMERA software (QED Imaging, Pittsburgh). At the end of all staining, cultures were incubated with Hoechst 33258 reagent for 10 min. BrdU staining was performed on ventral precursor cultures as previously described (19). All animal experiments were performed in accordance with the guidelines, ethical approval, and authorization of the Stockholm Norra Djurförsöks Etiska Nämnd (N65/03, N154/06, N88/07, and N145/09).

In Situ Hybridizations—Embryos were fixed (4% paraformaldehyde in phosphate-buffered saline at 4 °C) for 6 h, cryopreserved in 20% sucrose, frozen in OCT compound, and coronally sectioned onto slides (SuperFrostPlus). Wnt2 probe (559bp) was generated using Pvull restriction site (from nucleotide 349–908, NCBI number GI242397431:159–1241), and cloned into pCRII-TOPO, after adding dATPs to the blunt ends. T7 and SP6 RNA polymerases (Promega) were used to produce the RNA probes. ISH was performed as described previously (33). Briefly, ISH was performed on fixed tissue with digoxigenin (DIG)-labeled single-stranded RNA probes at 70 °C, followed by alkaline phosphatase-coupled anti-DIG antibody recognition and incubation with nitroblue tetrazolium (NBT) plus 5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrates. For immunohistochemistry (IHC), embryos were fixed in 4% PFA for 6 h, cryoprotected in 20% sucrose, and frozen in OCT com-
Purified Wnt2 Regulates Proliferation in the Midbrain

pound at −70 °C. Serial coronal sections of 14 μm were cut on a cryostat of the ventral midbrain. Following a brief 4% PFA post-fixation step, sections were incubated with the following antibodies diluted in PBS, pH 7.3, 1% bovine serum albumin, and 0.3% Triton X-100 solution: rabbit anti-Nurr1 (1:500, Santa Cruz Biotechnology, rabbit anti-TH (1:1000, Pel-Freeze Biologicals), and mouse anti-Ki67 (1:800, Abcam).

SN4741 Cell Culture and Immunoblotting—SN4741 cells were maintained and passaged as previously described (21). After stimulation with 40 ng of purified HA-Wnt2 for 2 h, SN4741 cells were harvested for Western blotting as described before (19). The proteins were transferred onto a polyvinylidene difluoride membranes, blocked in 5% nonfat dry milk, and incubated with the following primary antibodies: anti-active β-catenin (ABC, 1:500, Upstate/Millipore), anti-phospho LRP5/6 (pLRP5/6,1:500, Cell Signaling), (Dishevelled-2 (Dvl-2, 1:500, Santa Cruz Biotechnology). Appropriate secondary horseradish peroxide-conjugated antibodies were used, and signals were detected with ECL Plus reagent. For removal of the lipid modification, 50 ng of purified HA-Wnt2 was incubated with 19 ng of acyl-protein thiosterase-1 (APT-1) enzyme for 4 h at 30 °C and subsequently used to treat SN4741 as described above. Deglycosylation with N-glycosidase F was performed as previously described (21). For luciferase assays, 50,000 cells were plated in a 24-well plate and transfected with 50 ng of TOPFlash reporter constructs, and 10 ng of pRenilla as a control using Lipofectamine 2000 reagent (Invitrogen). Following 24 h of post-transfection, SN4741 cells were treated with purified Wnt2 overnight. Assays were performed using the dual luciferase reporter assay kit according to the manufacturer’s instructions (Promega).

RESULTS

Purification of Biologically Active Hemagglutinin-tagged Wnt2—The purification of Wnt2 was performed according to the protocol described by Willert et al. (31) using conditioned medium from stable Wnt2-overexpressing rat fibroblasts. Fractions eluted from each of the three respective purification steps were analyzed for the presence of HA-Wnt2 by immunoblotting with an anti-HA antibody (Fig. 1, A and B). Immunoblots and silver staining of eluted fractions showed a prominent band corresponding to the expected size of HA-Wnt2 (~41 kDa) (Fig. 1C and data not shown). The total amount of purified Wnt2 protein collected after the final purification step was low and estimated to contain ~3 ng/μl based on known standards of purified Wnt5a. The identity of the ~41-kDa band was also confirmed using an anti-Wnt2 antibody in eluted fractions that were also positive for HA immunoreactivity (Fig. 1D).

Wnt2 Activates β-Catenin in Dopaminergic Cells—To examine whether the purified protein was biologically active, we examined the ability of Wnt2 to activate Wnt signals in the SN4741 DA cell line. The SN4741 cell line was previously isolated from substantia nigra DA neurons of the developing mouse (E13.5, (34)) and has proven to be a faithful model for the characterization of Wnt signaling (35). We first sought out to determine whether purified Wnt2 affected β-catenin levels in DA cells. Increases in active (dephosphorylated) β-catenin (ABC) were observed following stimulation with Wnt2 (Fig. 2A). Further, treatment with Wnt2 also induced the phosphorylation of cytosolic proteins Dvl-2 and Dvl-3 as assessed by mobility shift (Fig. 2A and data not shown). Upon stimulation with Wnt2, phosphorylation of the Wnt co-receptor LRP5/6 was also detected (Fig. 2A). To assess whether Wnt2 signals activate TCF/Lef-mediated transcription, we performed TOPFlash luciferase reporter assays. Whereas unstimulated SN4741 cells had basal reporter activity, cells stimulated with 40 ng of Wnt2 resulted in a 2-fold increase in TOPFlash reporting activity (Fig. 2B). Expression of a stable, mutant form of β-catenin (S37A) increased TOPFlash activity 6-fold (Fig. 2B). These data suggest that Wnt2 activates Wnt/β-catenin signaling in DA cells.

Post-translational modifications by glycosylation and palmitoylation have been shown to be important for the biological activity of Wnts. We next addressed whether these modifications were critical for Wnt2 signaling. Upon addition of N-glycosidase F, an enzyme that removes asparagine-linked glycans, deglycosylation of Wnt2 failed to alter its native signaling abilities as assessed by Dvl phosphorylation and mobility shift (Fig. 2C). We also treated purified Wnt2 with acyl protein thiosterase-1 (APT-1), an enzyme that depalmitoylates Wnts and other substrates (31, 36) and found that Wnt2-mediated β-catenin dephosphorylation was abrogated (Fig. 2D). A similar decrease was also observed with Dvl-2/3 phosphorylation suggesting that the lipid modification of Wnt2 is required for its ability to trigger Wnt/β-catenin, whereas glycosylation is dispensable (Fig. 2, C and D).
Purified Wnt2 Increases the Number of Dopaminergic Neurons and Proliferating Ventral Midbrain Progenitors in Vitro—
To analyze the effects of Wnt2 on dopaminergic precursors, we treated E10.5 ventral mesencephalic precursor cultures with 30 ng of purified Wnt2. The differentiation of Wnt2-treated ventral midbrain precursors was assessed 3 days later by immunocytochemistry with the DA marker tyrosine hydroxylase (TH) and/or the early neuronal marker β-tubulin III (Tuj1). Interestingly, we observed an almost 2-fold increase in the number of TH-positive cells immunoreactive for TH (Fig. 3, A and B). Ventral progenitor cultures treated with Wnt2 displayed a ∼45% increase in the number of Tuj1+ neurons in vitro (Fig. 3C). To assess the specificity of this effect on DA neurons, we double-stained cultures with TH and Tuj1 antibodies. Upon treatment with Wnt2, precursor cultures exhibited a 40% increase in the proportion of neurons acquiring a DA fate (TH+/Tuj1+) (Fig. 3, A and D). In addition to the differentiation effects observed with Wnt2, we also assessed the effects of purified Wnt2 ligand on progenitor proliferation by measuring BrdU incorporation. Following a 2-h pulse prior to fixation, Wnt2-treated cultures displayed 2.5-fold increase in the percentage of cells incorporating BrdU label, compared with controls (Fig. 3E). These results suggested that Wnt2 increases the number of DA neurons in ventral midbrain precursor cultures by increasing neurogenesis.

Wnt2 Is Expressed in the Developing Ventral Mesencephalon—To examine the temporal expression of Wnt2 during DA differentiation, we performed qPCR analysis on mouse VM tissue from E10.5-E15.5. Wnt2 transcripts were detected at their highest levels at E10.5, and subsequently declined thereafter (Fig. 4A), suggesting an early function of Wnt2, supported by the in vitro data as well. We next examined the spatial distribution of these transcripts by in situ hybridization in E11 mouse embryos. As a control, Wnt2 expression was examined in the developing aorta, where it was detected at high levels, and in blood vessels where it is absent (Fig. 4B). Wnt2 expression was next examined in the midbrain and transcripts were detected at low levels throughout the ventral area of midbrain (Fig. 4B, ii and iii). Interestingly, higher levels of expression were detected in the intermediate and marginal zones of the ventral midbrain. Combined, our data indicate that Wnt2 expression is developmentally regulated and expressed in the ventral midbrain, suggesting a putative function for this ligand in regulating midbrain DA neuron development.

Decreased Progenitor Proliferation, DA Precursors, and DA Neurons in Wnt2-null Mice—We next examined the phenotypic consequences of Wnt2 deletion on developing DA neurons in vivo using Wnt2-null mice. Whereas deletion of Wnt1 was found to result in a segmental deletion of the midbrain-hindbrain regions, mice lacking one or both alleles of Wnt2 displayed no such defects and resembled wild-type animals (Fig. 5A) (14, 15). However, Wnt2-null mice displayed a modest, but significant decrease in the anterior-posterior length of the ventral midbrain length compared with control animals (Fig. 5B). It should be noted that most Wnt2-null animals die perinatally due to severe placenta and vascularization defects,
by the alteration at the level of proliferating progenitors, as suggested we examined the number of VM progenitors in the cell cycle, revealed no alterations (data not shown). However, when ventral midbrain for changes in the number of progenitors data suggested that targeted deletion of TH (Fig. 5) Nurr1, or their differentiation into TH (47) neurons was unchanged (Fig. 5, E), and that the defect was neurogenesis in the developing VM, allowing us to unravel a novel function of Wnt2 as a regulator of DA progenitor proliferation and neurogenesis, reducing thus the number of postmitotic cells (Nurr1+ progenitors and TH+ neurons) in the developing VM in vivo.

**DISCUSSION**

Studies on the function of Wnt2 in neural development have been hampered in the past by several factors, including (i) the lack of available Wnt2 protein to study Wnt2 signaling at a biochemical level, and (ii) early placentation and capillary defects in Wnt2-nulls (30), which dramatically reduced the number of pups available to analyze late phenotypes. Our study has addressed these two limiting factors by purifying Wnt2 protein and by extensive breeding to obtain the few Wnt2(+/−) mice presented in this study. We hereby describe the first reported purification of Wnt2 and its identification as an activator or Wnt/β-catenin signaling. Our findings demonstrate that purified Wnt2 activates canonical Wnt signals resulting in the accumulation of active β-catenin and phosphorylated Dvl2/3, as well as LRP5/6. Importantly these signals could be inhibited by removal of the lipid modification, underscoring the necessity of palmitoylation as an integral component of biologically active Wnt2, as it has been previously described for Wnt3a (31) and Wnt5a (21, 37). On the other hand, analysis of the neural phenotype of Wnt2 knockouts, in combination with precursor culture treatment with purified Wnt2 allowed us to unravel a novel function of Wnt2 as a regulator of DA neuron development. In particular, our findings identify Wnt2 as a regulator of DA progenitor proliferation and neurogenesis in the developing ventral midbrain. In this report, we found that Wnt2-null mice have fewer proliferating progenitors in the developing ventral mesencephalon and fewer postmitotic Nurr1+ and TH+ cells in the DA lineage. Accordingly, we found relatively high Wnt2 expression levels in intermediate and marginal zones but broad expression throughout the ventral midbrain, an expression pattern compatible with the observed regulation of proliferation and neurogenesis in Wnt2-nulls. These data, together with our in vitro results showing increased BrdU incorporation in progenitors and neurogenesis in VM precursor cultures treated preventing analysis at later stages (30). These defects also dramatically decreased the number of pups that could be obtained to perform this study. Upon further examination of the few embryos surviving at E11, we found that Wnt2-null mice exhibited a modest, but significant decrease (30%) in the number of TH+ DA neurons at E11 (Fig. 5, C and D), compared with control animals. We thus examined whether the decrease in the number of DA neurons could result from a defect in the number of DA progenitors expressing the nuclear receptor Nurr1, or their differentiation into TH+ neurons. Interestingly, we found that while the number of Nurr1+ cells was decreased (Fig. 5E), the proportion of Nurr1+ progenitors that matured into TH+ dopamine neurons was unchanged (Fig. 5F). These data suggested that targeted deletion of Wnt2 had no effect on DA precursor differentiation in vivo and that the defect was upstream.

We then examined whether the decrease in the number of Nurr1+ cells in Wnt2 (−/−) mice could be attributed to an alteration at the level of proliferating progenitors, as suggested by the in vitro data. Examination of the ventricular zone of the ventral midbrain for changes in the number of progenitors revealed no alterations (data not shown). However, when we examined the number of VM progenitors in the cell cycle, as detected by Ki67 staining, we found a 36% decrease in the number of proliferating cells in Wnt2-null embryos compared with control animals (Fig. 5, G and H). These results suggest that the loss of Wnt2 decreases progenitor proliferation and neurogenesis, reducing thus the number of postmitotic cells (Nurr1+ progenitors and TH+ neurons) in the developing VM in vivo.
with Wnt2, indicate that Wnt2 primarily regulates proliferation and neurogenesis in the ventral midbrain progenitors.

Several reports have highlighted the importance of Wnt signals in developing dopaminergic neurons. Expression analysis has previously shown that in addition to Wnt1 and Wnt5a, Wnt2 is expressed at relatively high levels in the developing VM (19, 20). Similarly, several Frizzled receptors and co-receptors are highly expressed in the ventral midbrain, including Fz5 and Fz9 (20, 38), indicating that distinct functional receptors and pathways can be activated by several Wnts. In agreement with this data, we and others have shown that different Wnts serve specific functions in midbrain dopaminergic neuron development. We previously found that purified Wnt5a increases the proportion of DA precursors that differentiate into midbrain DA neurons in vitro (21). Genetic analysis of Wnt5a function in vivo, indicated that Wnt5a-null mice display a transient increase in VM progenitor proliferation and differentiation resulting in an excess of Nurr1+ precursors that fail to differentiate into TH+ cells (6). In contrast to the effects observed in Wnt5a(+/−) mice, Wnt1-null mice exhibit a partial deletion of the midbrain and DA deficits that are a consequence of altered patterning and specification (explained by a loss of Otx2 expression in the DA progenitor domain), and compromised DA neuron survival (loss of Pitx3 expression) (16). The phenotype described in Wnt2-null animals is overall distinct from both Wnt1 and Wnt5a knock-out mice but exhibits some features that may resemble the function of Wnt1. Unlike Wnt5a(+/−) embryos, no increase in progenitor proliferation or decrease in dopaminergic differentiation (%TH+/Nurr1+ cells) was detected. In contrast, Wnt2(+/−) mice displayed a decrease in the proliferative capacity of DA progenitors in the ventricular zone of the VM and in their capacity to undergo neurogenesis, as indicated by the reduction of postmitotic cells (Nurr1+ and TH− cells) in the DA lineage. Wnt1 has also been described to regulate progenitor proliferation in the developing VM (17, 19) as well as the number of Nurr1 and TH+ cells in the VM (16, 18, 19). However, other features of the Wnt1(+/−) mutant were not phenocopied by the Wnt2(+/−) animals, indicating that each Wnt regulates a specific combination of functions, some of which are redundantly regulated by distinct Wnts. These results point to a level of complexity that is greater than expected from the activation of canonical versus non-canonical pathways. This level of complexity could be accomplished by the known expression patterns of different Wnts and their receptors, which would lead to the activation of different Wnt signaling pathways in specific domains and windows of time, permitting unique, highly context-dependent interactions. One such interaction could be brought about by Fz5, a receptor shared by both Wnt2 and Wnt5a (39). Interestingly, Fz5 was found to synergize with either Wnt2 or Wnt5a in secondary axis induction assays in Xenopus oocytes. This finding is very interesting as it suggests a potential cross-talk between β-catenin-mediated signals (activated by Wnt2) and β-catenin-independent signals (activated by Wnt5a) in the developing ventral mesencephalon. In support of this, we recently reported that components of canonical Wnt signaling, such as Lrp6, also regulate the activation of non-canonical Wnt signaling (Rac1 activity) by Wnt5a (40). This crosstalk was conserved from Xenopus to mice, where deletion of Wnt5a completely rescued the exencephaly in Lrp6(−/−) mice (40). In agreement with the idea of cross-talk, Fz5-null mice display proliferation defects...
which largely phenocopy Wnt2 mice (39, 41). These results, together with the work described here and the known functions of Wnt5a in DA neuron development, suggest that receptors like Fz5 may be critical for the integrity of both canonical and non-canonical Wnt pathways during midbrain DA development. Whether Wnt2 and Wnt5a signals act separately or impinge on one another to induce DA differentiation remains to be determined. Future experiments examining conditional Wnt2/Fz5 or Wnt5a/Fz5 compound mutants should provide insight into the possible physiological function of these ligand/receptor pairs in DA development.

Acknowledgments—We thank Drs. Clare Parish and Helder André for critical reading of the manuscript and Claudia Tello for additional assistance. We are grateful to Jan Kitajewski for providing Wnt2-overexpressing fibroblasts. We thank Johny Söderlund, Alessandra Nanni, and the Arenas laboratory members for assistance and fruitful discussions. We thank Stephen Byers (Georgetown University) for the S37A β-catenin construct.

REFERENCES
1. Nusse, R. (2005) Cell Res. 15, 28–32
2. Chenn, A., and Walsh, C. A. (2002) Science 297, 365–369
