Wnts guide longitudinal axon tracts in the brain

Doctoral Thesis

Asheeta Ajashni Prasad

ISBN : 978-90-8891-356-3

The printing of this thesis was partly funded by Zeiss Microscope, Rudolf Magnus Institute and J.E.Jurriaanse Stichting

Printing by Printyourthesis.com

The cover was inspired by the subject of this thesis, where different parts of the brain are connected by axons. This concept is represented by the various continents. The images used here were collected by Asheeta Prasad during her Phd. Carlyn Mamber compiled the images artistically.

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Wnts sturen longitudinale zenuwbanen in de hersenen
(met een samenvatting in het Nederlands)

Proefschrift
ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op
maandag 5 december 2011 des ochtends te 12.45 uur

door

Asheeta Ajashni Prasad

geboren op 26 september 1980
Lautoka, Fiji eilanden

Promotor:	Prof. dr. J.P.H. Burbach
Co-promotor:	Dr. R.J. Pasterkamp

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Chapter 1

Introduction

Preface

The brain is composed of a network of neuronal connections essential for the control of numerous physiological functions. The establishment of these connections during development requires embryonic axons to extend through the extracellular environment to their synaptic targets. This process of axon guidance is mediated by molecular cues in the extracellular matrix known as axon guidance molecules. Longitudinal axonal tracts such as the striatonigral, striatopallidal, dopaminergic and serotonergic pathways are exquisitely complex and require a myriad of molecular signals for their proper development. Surprisingly little is known about the molecular events that underlie the establishment of these pathways. Wnt proteins are secreted molecules generally expressed in gradients along the anterior-posterior (A-P) axis of the developing nervous system. Wnts are known to serve as axon guidance signals for afferent and efferent spinal cord projections. This thesis explores a novel role for Wnts and their receptors in the A-P guidance of striatonigral, striatopallidal, nigrostriatal, and serotonergic axons.

Neural Circuit Development

The human brain contains more than 10 billion neurons that form over 10 trillion connections. This complex pattern of connectivity begins to form during embryonic development, when newly born neurons grow and extend processes to regions in and outside the brain. Neural circuit development requires several fundamental cellular events including neuron polarization, axon and dendrite growth and guidance, target recognition and synapse formation.

In this thesis the focus is on how axons navigate through the extracellular matrix (ECM) to find their synaptic targets, a process known as axon guidance. A cellular structure essential for axon guidance is the growth cone, a sensory apparatus at the tip of the axon. The growth cone senses and responds to axon guidance cues in the ECM. Axon guidance molecules can either induce attractive or repulsive growth cone responses and they can be secreted or membrane-bound. The classical axon guidance families are formed by the Netrins, Slits, Semaphorins, ephrins/Ephs, and repulsive guidance molecules (RGMs) (Chisholm and Tessier-Lavigne, 1999) (Fig. 1)

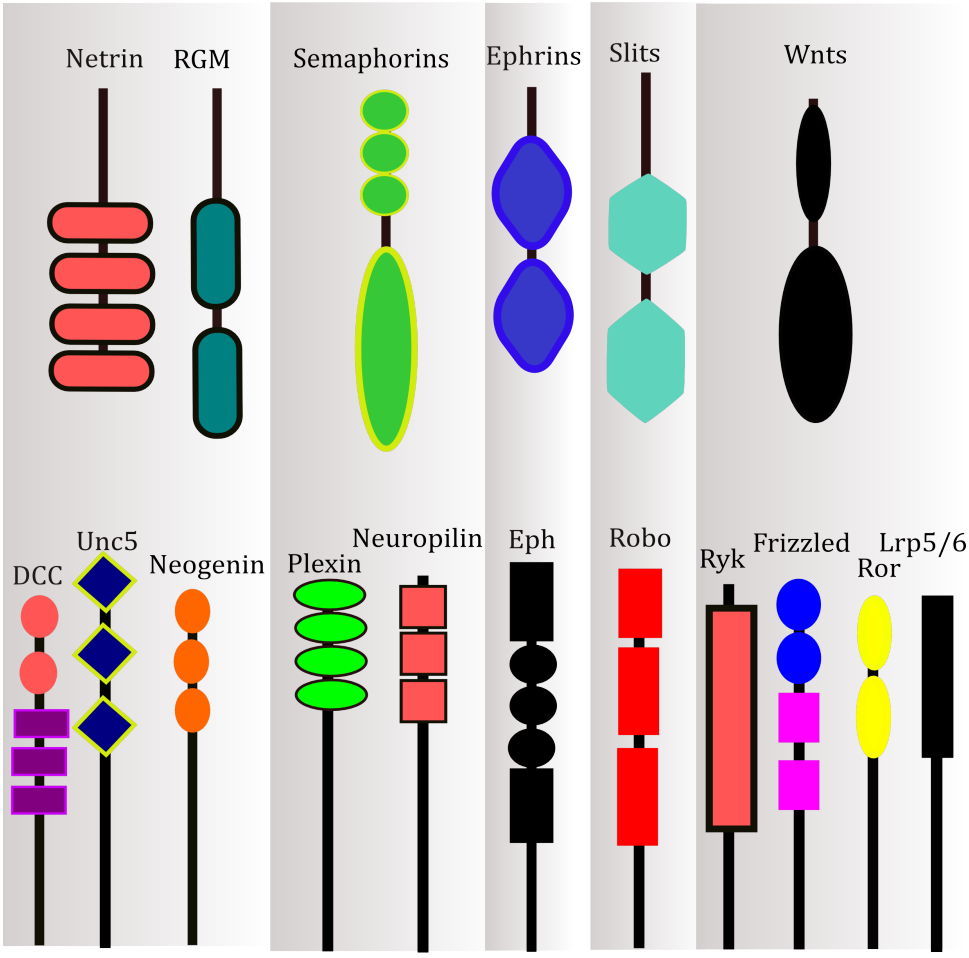

Figure 1: Axon Guidance Molecules and their Receptor complexes.
Netrins signal via Deleted in Colorectal Cancer (DCC), UNC5 and Neogenin receptors. RGM also signals via Neogenin and UNC5. Slits bind to roundabout (Robo) receptors. Ephrins bind to Eph receptors, and vice versa. Semaphorins can bind to Plexin and Neuropilin receptors. Wnt molecules interact with Frizzled, Lrp5/6, Ryk and Ror receptors. It should be noted that more proteins with axon guidance effects have recently been identified. For this thesis, we focus on the largest and best-characterized families. Each of the families indicated above contains multiple different members.

The sensitivity of a growth cone to axon guidance cues is determined by its expression of specific receptors (Dickson and Zou, 2010) (Fig. 1). Netrins signal via Deleted in Colorectal Cancer (DCC), UNC5 and Neogenin receptors (Livesey FJ, 1999). Slits exert their repulsive effects through roundabout (Robo) receptors (Nguyen-Ba-Charvet and Chedotal, 2002). Ephs are ephrin receptors but, vice versa, ephrins can also act as Eph receptors (Du et al, 2007). Semaphorins bind to Plexins and/or Neuropilins to induce repulsion or attraction (Zhou et al, 2008). And finally, repulsive guidance molecules (RGMs) use Neogenin and UNC5 for mediating axon guidance (Yamashita et al, 2007). In addition to these classical axon guidance molecules, morphogens such as Wnts have been shown to act as guidance proteins. Wnt molecules are highly versatile axon guidance cues, involved in attraction, repulsion but also axon outgrowth. The functions of Wnts are mediated through combinatorial interactions between Frizzled, Lrp5/6, Ryk and Ror receptors (Charron and Tessier-Lavigne, 2005).

The developing brain is divided into neuromeres, in the forebrain these are called prosomeres and in the hindbrain rhombomeres (Fig. 2). Neuromeres are organized along the A-P axis of the brain and several axon tracts project along this axis. Examples include axons from striatonigral, striatopallidal, mesodiencephalic dopaminergic (mdDA), and serotonergic (5HT) neurons.

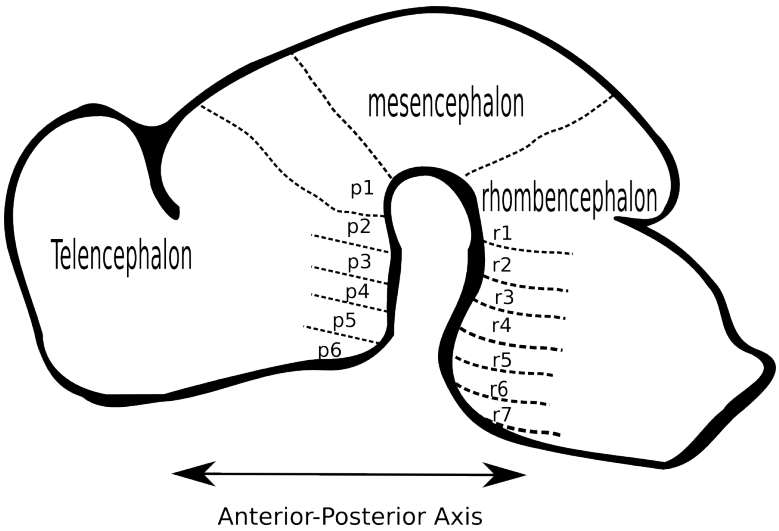

Figure 2: Anterior-posterior organization of the developing brain.
The developing brain is organized along the anterior-posterior (A-P) axis in segmented structures known as neuromeres. r = rhombomere, p = prosomere.

During development, these axons travel over long distances and require a multitude of axon guidance cues to reach and innervate their final targets. The molecular mechanisms required for the patterning of longitudinal axon tracts along the A-P axis are poorly understood. This thesis aims to fill this void by focusing on the axon guidance mechanisms involved in the development of four major axon tracts between the forebrain and the mid/hindrain (the striatonigral, striatopallidal, mdDA, and 5HT tracts). In the following sections the function, anatomy and development of these axon tracts will be summarized and discussed.

Striatum

The striatum is a brain region crucial for motor control, motivation and cognitive behaviors. Aberrations in striatal circuitry are implicated in movement disorders such as Huntington's disease, Parkinson's disease, Tourette's and Dystonia. In addition, psychiatric disorders such as depression and obsessive and compulsive disorder (OCD) involve the striatum and its projections (Gerfen et al, 1992).
Approximately 95% of all neurons in the striatum are medium spiny neurons (MSNs). The remaining 5% are aspiny interneurons. MSNs are projection neurons with targets in more caudal parts of the brain and express g-aminobutyric acid (GABA) as their main inhibitory neurotransmitter. MSNs are born in the lateral ganglionic eminence (LGE) of the ventral telencephalon (Hamasaki et al, 2003). Postmitotic neurons migrate laterally from the LGE to the striatal mantle zone to terminally differentiate into mature striatal neurons (Wilson and Rubenstein, 2000). In mice, MSNs are generated between E12.5 and E15.5 (Jain et al, 2001). It has been well established that the birth stage of striatal neurons

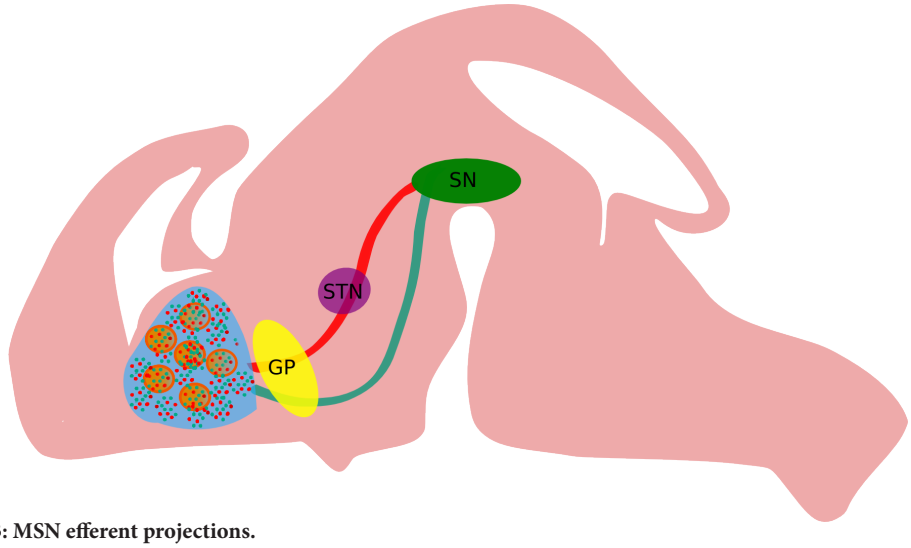

Figure 3: MSN efferent projections.
MSN neurons (red and teal dots) are distributed in the striatum (blue region); a subset of neurons is organized in patches known as striosomes (orange circles) and the surrounding neurons form the matrix. Striatonigral axons (teal) project directly to the substantia nigra (SN). Striatopallidal neurons project to the globus pallidus pars externa (GPe; yelow). GPe neurons then send axons to the subthalamic nucleus (STN; purple) and the STN projects to the SN.

determines their compartmentalization to either the matrix or the striosome parts of the striatum (van der Kooy and Fishell, 1987). Early born striatal neurons accumulate together to form the striosome, also known as patches (Song and Harlan, 1994). The later migrating neurons form the matrix. Approximately 80-85% of all MSNs form the matrix and are distributed around the striosomes (Jain et al, 2001, Passante et al, 2008) (Fig. 3). MSNs (both in the striosome and matrix) segregate into two populations called striatonigral and striatopallidal neurons based on their axon projections and gene expression patterns. In striatonigral neurons, dopamine receptor D1, muscarinic receptor M4 and tachykinin 1 are exclusively expressed. Striatopallidal neurons express preproenkephalin 1, dopamine receptor D2 and adenosine receptor A2a (Lobo et al, 2007). These two different populations of MSNs give rise to two axonal bundles, the striatonigral and the striatopallidal tracts. Striatonigral axons project directly to the substantia nigra pars reticulata (SNr). Striatopallidal axons travel a much shorter distance and synapse in the globus pallidus pars externa (GPe) contacting neurons that give rise to connections from the GPe to the subthalamic nucleus (STN). STN axons then project to the SNr. The two efferent striatal tracts are commonly known as the direct and indirect pathways based on their route to the SNr. Both tracts provide inputs into the SNr thereby affecting motor control (Brazhnik et al, 2008, Gerfen, 1992). The pathways work antagonistically, i.e. the direct pathway promotes movement and the indirect pathway inhibits movement (Graybiel, 2000). So this network can exert a well-balanced control over movement.

Axon guidance of Medium Spiny Neurons

It remains largely unknown how MSNs, intermingled in the striatum, are able to form two distinct axon pathways to the SNr. So far members of the Semaphorin and Protocadherin protein families have been implicated in the guidance of MSN axons. However, given the complexity of both pathways many more molecular cues must be involved in their development.

Semaphorins

Semaphorin 3E (Sema3E) is a secreted axon guidance molecule belonging to the Semaphorin family (Fig. 1). Semaphorins signal via Plexin and/or Neuropilin receptors (Zhou et al, 2008). PlexinD1 is expressed on developing striatonigral axons and binds to Sema3E present in the extra cellular matrix (ECM). In the absence of Sema3E or plexinD1, striatonigral axons are misrouted into regions they normally avoid and that express Sema3E (i.e. the thalamic reticular nucleus and dorsal midbrain). Thus, Sema3E and plexinD1 work together in the pathfinding of striatonigral axons (Chauvet et al, 2007).

Protocadherins

Protocadherin 10 (Ol-pc) is a member of the protocadherin family (Uemura et al, 2007). Ol-pc is expressed on striatal axons and is essential for their outgrowth *in vitro* and *in vivo*. *Protocadherin 10* (*Ol-pc*) knockout mice show severe defects in various axonal tracts running through the ventral telencephalon. Moreover, the patterning of the GP and of corridor cells (López-Bendito et al, 2006) is perturbed in *Ol-pc* deficient mice. Based on these findings it has been proposed that Ol-pc promotes the growth of striatal axons thereby indirectly influencing the development of other forebrain tracts.

Mesodiencephalic dopaminergic system

Mesodiencephalic dopamine neurons (mdDA) neurons acquire their name from their origin within the mesencephalon and diencephalon and from their characteristic expression of the neurotransmitter dopamine. mdDA connectivity has been implicated in motor control, motivation and reward seeking behavior (Smidt and Burbach, 2007). Defects in the dopaminergic function and connectivity are associated with several neural diseases including schizophrenia and Parkinson's disease (Riddle and Pollock, 2003).
Anatomically distinct groups of mdDA neurons have been identified in the substantia nigra pars compacta (SNc or A9), the ventral tegmental area (VTA or A10) and Retrorubral Field (RRF or A8) (Björklund and Dunnett, 2007). mdDA neurons originate from progenitors from the ventricular zone and differentiate during migration to lateral positions to mature into mdDA neurons. They first appear at E14. Between E14-16 most mdDA neurons have reached their final positions.

Three main mdDA axonal tracts have been described, the mesostriatal, mesocortical and mesolimbic pathways (Björklund and Dunnett, 2007). They bundle together throughout the diencephalon and segregate in the telencephalon. SNc neurons project axons to the dorsal striatum forming the mesostriatal pathway. Distinct from SNc projections, VTA and RRF axons prominently innervate the ventromedial striatum and prefrontal cortex (PFC), contributing to the mesolimbic and mesocortical pathways, respectively.

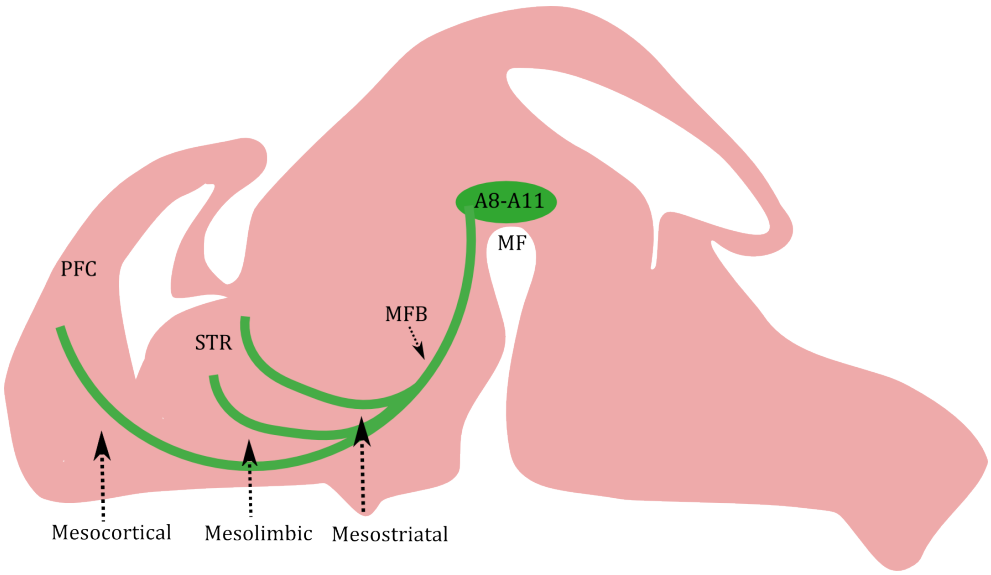

Figure 4: mdDA axon pathways.
mdDA neurons (green) are located at the mesencephalic flexure (MF) and extend projections rostrally towards the forebrain. mdDA axons fasciculate together forming the medial forebrain bundle (MFB). mdDA projections to the dorsal striatum form the mesostriatal tract. Connections to the ventral striatum and prefrontal cortex are known as the mesolimbic and mesocortical pathways, respectively.

mdDA projections initially follow a dorsal trajectory within the mesencephalon but then deflect rostrally towards the diencephalon (Namakura et al, 2000). At E13.5, mdDA axons take a ventral turn along the A-P axis. As they traverse the diencephalon individual mdDA axons fasciculate into two large ipsilateral axon bundles, called the medial forebrain bundles (MFBs). By E14, mdDA fibers in the MFB reach and start to invade the area ventrolateral to the ganglionic eminence (GE) (Kolk et al, 2009). By E18.5, distant targets such as the striatum and cortex are prominently innervated by mdDA axons. The development and refinement of dopaminergic connectivity proceeds during the first weeks after birth (Van den Heuvel and Pasterkamp, 2008).

Axon guidance of mesodiencephalic dopamine (mdDA) neurons

Several studies have indicated roles for different axon guidance families in the formation of mdDA pathways. A brief summary of the most recent findings is given here, but more details are provided in Chapter 2.

Semaphorins and Neuropilin/Plexin

Semaphorins have been implicated during several stages of mdDA circuit formation. Repulsive Sema3F and neuropilin-2 (Npn2) signaling is required for the stereotypic rostrally oriented growth of mdDA axons (Yamauchi et al, 2009) and for the fasciculation of the MFB (Kolk et al, 2009). Interestingly, Sema3F is a bi-functional guidance cue and in the prefrontal cortex acts as an axon attractant cue. It attracts Npn2 expressing mdDA axons into the developing prefrontal cortex (Kolk et al, 2009).

Ephrin/Eph

Eph and ephrins have been implicated in the formation of topographic connections between the mdDA system and the striatum. EphB1 is expressed in the SN, but not or weakly in the VTA. In the embryonic striatum, the repulsive EphB1 ligand ephrinB2 is strongly expressed in the ventromedial striatum (targeted by VTA axons) but only weakly in the dorsolateral striatum (targeted by SN axons) (Hu et al, 2004). It should be noted, however, that analysis of *EphB1* mutant mice is incoherent with the idea that EphB1 functions in the topographic mapping of mesostriatal connections (Richards et al, 2007). This hints at compensation by other Eph/Ephrins expressed in the mesostriatal system such as ephrin-B3 and ephrin-A5 (Sun et al, 2010 , Cooper et al, 2008).

Slit/Robo and Netrin/DCC

Slits are potent chemorepulsive cues. Slit1 and Slit2 are expressed around the mesencephalic flexure and mdDA neurons express the Slit receptors Robo1 and Robo2. Slit2 robustly repels embryonic mdDA axons in collagen matrix assays (Dugan et al, 2011). Furthermore, mdDA axons exhibit pathfinding errors in *Slit1/2* and *Robo1/2* knockout mice (Dugan et al, 2011). In zebrafish, Slit/Robo2 and Netrin/DCC are expressed along the midline. It is hypothesized that the attractive forces of Netrin are needed to keep dopaminergic axons close along the midline and that repulsive Slit signals to ensure that mdDA axons do not cross the midline (Kastenhuber et al, 2009).

Morphogens

Morphogens such as Wnts and Sonic hedgehog (Shh) are essential during the early patterning and specification of mdDA neurons (Smidt and Burbach, 2007). Only recently they have also been implicated in mdDA axon guidance. Shh acts as a chemoattractant for mdDA axons in explant assays and in *Shh* deficient mice medial mdDA projections are misrouted and reduced (Hammond et al, 2009). The role of Wnts in mdDA axon guidance is described in Chapters 3, 4 and 5 of this thesis.

Serotonergic system

Serotonergic (5HT) neurons, like dopamine neurons, are named based on their neurotransmitter expression, which is serotonin (Goridis and Rohrer, 2002). Defects in serotonergic connectivity have been implicated in neural diseases such as depression and autism (Daubert and Condron, 2010).

The total number of serotonergic neurons in the adult rat is approximately 20,000 (Jacobs et al, 1992). Serotonergic neurons are born in the ventral rhombencephalon and are clus tered in nine groups (B1–B9). As early as E10 in mice, serotonergic destined neurons are detectable. Time-lapse images show that 5HT neurons migrate via somal translocation to their final targets in the brainstem (Hawthorne et al, 2010). In the adult, the rostral B6 group of 5HT neurons will line the raphe nuclei; B7 neurons localize in the dorsal raphe and B8 neurons in the median raphe. The caudal groups of 5HT neurons (B1-B3) will eventually occupy the magnus, obscurrus, and pallidus raphe nuclei (Gaspar et al, 2003).

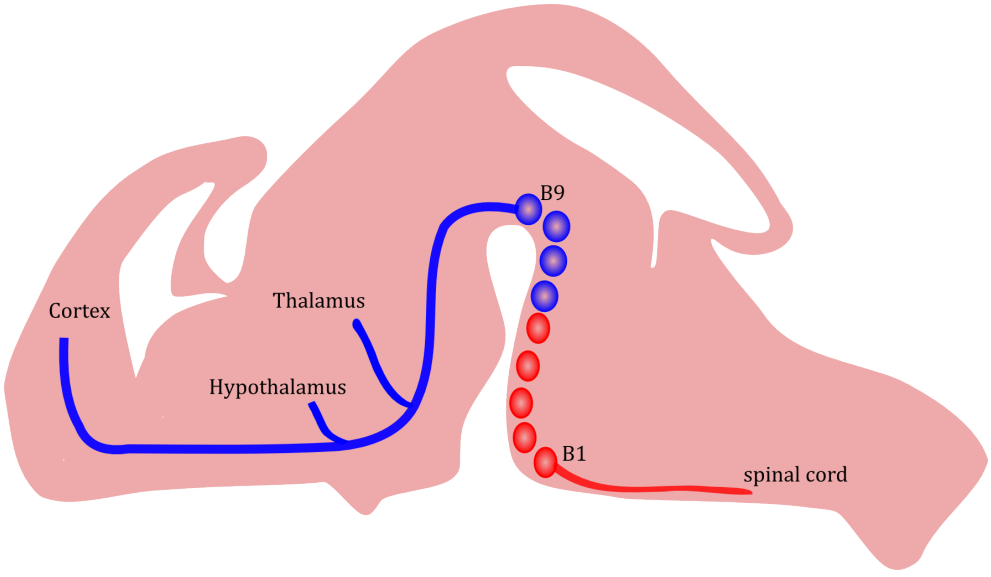

Figure 5: Serotonergic projections.
Serotoninergic neurons are divided into 9 subgroups, B1-B9. Serotonergic neurons in B6-B9 (blue) are located rostral to the isthumus in rhombomeres 1-3 and form ascending projections to the midbrain and the forebrain. Neurons in the B1–B5 (red) groups are located in rhombomeres 5-9 and form descending projections to the spinal cord.

5HT neurons start to extend axons at E11.5 and by E15 ascending projections that originate from neurons in the B6-B9 groups reach the diencephalon. From here, these 5HT fibers merge with other axonal tracts such as the fasciculus retroflexus and the MFB to reach their forebrain targets (Gaspar et al, 2003). Ascending 5HT fibers project to the telencephalon and the hypothalamus by E17. Descending axons, from neurons in the B1–B5 group project to the brainstem and spinal cord innervating preganglionic sympathathetic and somatic motor neurons. Completion of the serotonergic network occurs with axonal terminal branching at postnatal stages (Gaspar et al, 2003)

Axon guidance of serotonergic neurons

Serotoninergic axons virtually innervate the entire brain (Gaspar et al, 2003). However, our current understanding of the cues involved in the formation of 5HT pathways is rather limited. The cortex, striatum and ventral mesencephalic regions are all innervated by 5HT axons. Explants from these three targets show massive innervation by 5HT axons when co-cultured with 5HT explants (Petit et al, 2001). This indicates a role for short-range, membrane-bound cues in the guidance of 5HT axons. In line with this idea, glycosylphospatidylinositol (GPI)-linked membrane proteins have been shown to promote 5HT axon outgrowth (Petit et al, 2005). When astrocytes are implanted into the adult rat cortex, striatum or ventral mesencephalon, a prominent innervation of the transplantation site by 5HT axons can be detected (Petit et al, 2001). It has therefore been hypothesized that astrocytes express axon growth and guidance cues for 5HT axons.

Wnt/Frizzled signaling in the Central Nervous System

Wnt/Frizzled signaling has been implicated in various aspects of neuronal network development (Salinsa and Zou, 2008). For example in the developing brain, Wnt proteins are often expressed in gradients along the A-P axis and are required for A-P guidance of axons in the spinal cord (Lyuksyutova et al, 2003, Liu et al, 2008). This makes Wnts strong candidates for regulating the A-P guidance of the axonal pathways studied in this thesis.
Wnts are a large family of secreted proteins with 19 members in vertebrates (Miller JR, 2002). Wnt proteins control different stages of neuronal development such as neuronal specification/differentiation (McMahon and Bradley, 1990), axonal outgrowth (Li et al, 2009), axonal guidance (Keeble et al, 2006, Zaghetto et al 2007), and synapse formation (Davis et al, 2008). Their pleiotropic effects are the result of interactions with different receptors and the activation of different downstream signaling mechanisms. Wnts are able to bind to Frizzled (Fzd), low-density lipoprotein receptor (Lrp5/6), Ryk and Ror receptors.
The Fzd family of Wnt receptors includes 10 members (Fischer et al, 2007). Fzds are seven trans-membrane proteins and their N-terminal part, which is exposed to the extracellular environment, contains a cysteine-rich domain (CRD) used for Wnt binding (Chen et al, 2004). Wnt/Fzd can signal via Canonical, Non-canonical or Planar cell polarity pathways (PCP). Fzd receptors are central to Wnt signaling because they are involved in all the Wnt signaling pathways discussed below.

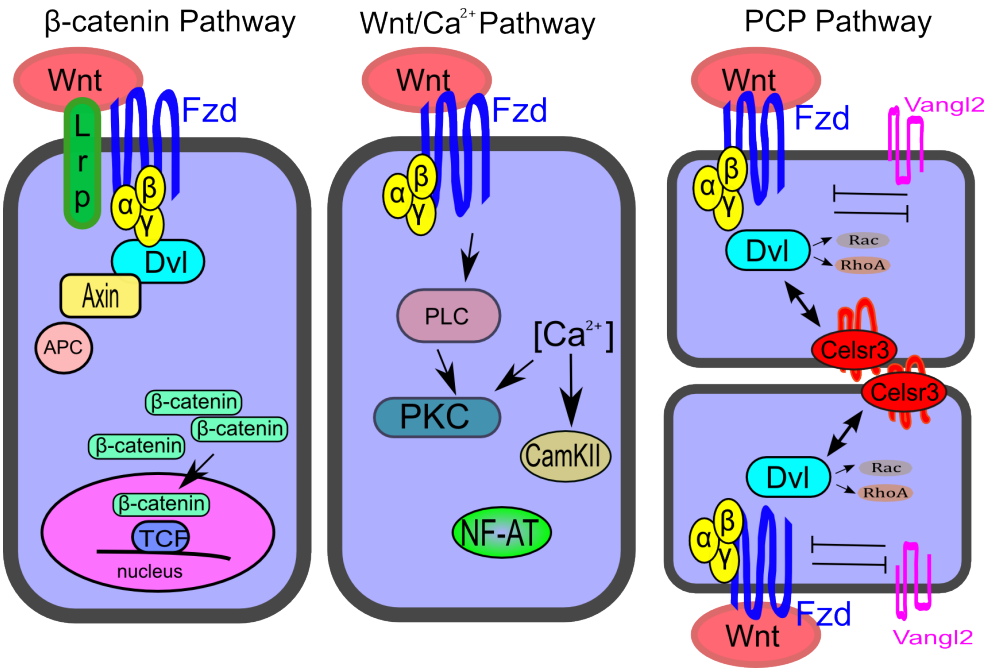

Figure 6: Wnt signaling cascades.
Canonical pathway: Wnt binding induces the formation of a complex between Fzd and Lrp5/6 activating Dishevelled. This results in the disassembly of the "destruction complex" that normally degrades ß-catenin, allowing translocation of ß-catenin into the nucleus where it regulates gene transcription. Wnt/Ca $^{2+}$ pathway: Wnt/Fzd binding increases the intracellular concentration of Ca^{2+}, initiating a cascade of intracellular signals leading to the transcription of NFAT-responsive genes. PCP pathway: Activation of Dishevelled via PCP receptor complex regulates Rho/Rac GTPases to control cytoskeletal dynamics.

Canonical pathway

In the canonical pathway, Wnt protein binding to a Frizzled receptor initiates receptor heterodimerisation with the low-density lipoprotein receptor-related protein (Lrp5/6) (Ciani & Salinas 2005). Lrp5/6 are single transmembrane proteins specifically required as co-receptors in the canonical pathway. The receptor complex activates the cytoplasmic protein Dishevelled leading to the disassembly of the destruction complex (Axin/ adenomatis polysis coli (APC) and glycogen synthase kinase 3β), which in the absence of Wnt binding degrades β-catenin. A key feature of the canonical pathway is the translocation of β-catenin to the nucleus. In the nucleus, β-catenin activates transcription factors such as TCF (T-cell specific transcription factor) and LEF (lymphoid-enhancing factor). As a result of this activation, the expression of a large set of genes with functions in cell fate decisions, microtubule rearrangement, axon guidance, and synaptogenesis will be regulated (Li et al, 2010).

Wnt/Ca^{2+} pathway

The downstream effect of Wnt/Ca^{2+} signaling is an increase in calcium ions (Ca^{2+}) in the plasma membrane. In this pathway, the binding of Wnt to a Fzd receptor leads to the activation of Disheveled. This in turn increases intracellular Ca^{2+} levels. Elevated levels

of Ca^{2+} antagonize β-catenin and allow the nuclear translocation of nuclear factor of activated T-cell (NFAT), initiating transcription of NFAT-responsive genes (Slusarski and Pelegri, 2007). The role Wnt/Ca^{2+} signaling has been recently shown in development of the mouse corpus callosum. For chemorepulsion of callosal axons, Wnt5a binding to the Ryk receptor opens inositol 1,4,5-trisphosphate receptors and Transient receptor potential channels causing release of Ca^{2+} from intracellular stores, resulting in the influx of Ca^{2+} in the plasma membrane. This short outburst of Ca^{2+} activates Calmodulin Kinase II to accelerate axon extension and growth cone repulsion (Hutchins et al, 2011).

Planar cell polarity

The Planar cell polarity pathway (PCP) also requires Fzd and two other core PCP receptors, cadherin EGF LAG seven pass G-type receptor 3 (Celsr3) and Vang-like 2 (Vangl2). In response to Wnt binding to these core PCP receptors, Disheveled is activated. This initiates the activation of c-Jun N-terminal kinase (JNK) and small GTPases (of the Rho family). These intracellular signaling cues regulate cytoskeleton dynamics. Planar cell polarity is the process involved in assembling the orientation of asymmetric structures within a plane (Wang et al, 2006). In vertebrates, PCP pathway is required for the neural tube formation, where the ectoderm undergoes mediolateral convergence and elongates along the A-P axis (Zou , 2004)

Other Non-canonical signaling events (in) dependent of Fzd have also been reported. Wnts can for example interact with Ryk and Ror receptors. Ryk is a tyrosine kinase receptor and Wnt proteins are the only known ligands for Ryk. Ryk-Wnt interactions can induce both canonical and non-canonical signaling (Lu et al, 2004). Ryk-Wnt signaling is involved in axon guidance and mediates chemorepulsive guidance responses. Major defects are detected in the formation of the corticospinal tract and the corpus callosum in *Ryk* mutant mice (Schmitt et al, 2006 and Keeble et al, 2006). Interestingly, the intracellular domain of Ryk can be cleaved which is then translocated to the nucleus to induce neuronal differentiation (Lyu et al, 2008).

Ror receptors are the most recent addition as Wnt receptors (Masiakowski and Carroll, 1992). In vertebrates, there are two Receptor tyrosine kinase-like orphan receptors, Ror1 and Ror2. The extracellular CRD of Ror is similar to the Wnt-binding domain in Fzd receptors (Saldanha et al, 1998). This strongly suggests Ror receptors are capable of binding Wnts (Oishi et al, 2003). Wnt/Ror activation is involved in neurite outgrowth, axon guidance, migration and morphogenesis (Song et al, 2010; Kennerdell et al, 2009; Lyashenko et al, 2010)

Defects in MSN, mdDA and 5HT system have been strongly implicated in Parkinson's disease, depression and drug addiction. Yet very little is known about their molecular development (as summarized above).

Wnt proteins have very versatile biological roles. Along with their expression at the A-P axis makes them intriguing candidates to study in the development of these longitudinal tracts. This study can contribute to the design of therapeutics to repair loss of neuronal connectivity in MSN, mdDA and 5HT systems.

Aim and Outline of Thesis

Longitudinal axon tracts such as those originating from MSN, mdDA and 5HT neurons require a multitude of molecular signals for navigating the developing brain en route to their synaptic targets. Unfortunately, relatively little is known about the signals involved in the development of the striatonigral, striatopallidal, dopaminergic and serotonergic pathways. Furthermore, the molecular cues that regulate the growth and guidance of these tracts along the A-P axis of the brain remain to be identified. In the past several years, Wnts have emerged as A-P guidance cues for spinal cord afferent and efferent projections. The overall aim of this thesis was to examine the role of Wnts and their receptors in the A-P guidance of longitudinal axon tracts in the forebrain (i.e striatonigral, striatopallidal, dopaminergic and serotonergic pathways). This strategy was taken to better understand the molecular makeup of these vital axonal tracts.

Chapter 2
Axon guidance in the dopamine system.

This chapter describes the development of mdDA projections in the telencephalon and summarizes the axon guidance molecules involved in the path finding, target innervation, and pruning of these axons.

Chapter 3
Wnt/planar cell polarity signaling controls the anterior-posterior organization of monoaminergic axons in the brainstem

This chapter for the first time reveals a role for Wnts in A-P guidance outside the spinal cord. Wnts and several PCP receptors are found to cooperate in the patterning mdDA and 5HT axons in the developing brainstem.

Chapter 4
Frizzled-3 regulates the topology of the developing mesodiencephalic dopamine (mdDA) system

Previously Wnts have been reported to control the development of the mdDA neuron pool. This chapter identifies Fzd3 as an important receptor mediating such Wnt effects. Fzd3 is found to be crucial for the medio-lateral positioning of mdDA neurons.

Chapter 5
A role for Wnt5a/Ryk signaling in the formation of mesostriatal circuitry?

In chapter 3, Wnt5a was identified as a chemorepellent for mdDA axons through binding to Fzd3. In chapter 5 the role of Ryk in this Wnt5a-mediated axon repulsion is studied in culture and *in vivo* in mouse models.

Chapter 6
Fzd3 is required for the formation of the striatonigral and striatopallidal pathways

In this chapter, the role of Fzds in the formation of axon projections from MSN in the striatum is explored. Fzd3 is shown to be required for the development of both the striatonigral and striatopallidal pathways.

Chapter 7
General Discussion

Reviews the overall findings of this thesis; evaluating the characteristics of Wnt/Fzd signaling and their ability to exert axon guidance roles in during the formation of striatonigral, striatopallidal, mdDA, and 5HT connections.

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Chapter 2

Axon guidance in the Dopamine System

Asheeta A. Prasad and R. Jeroen Pasterkamp

Development and Engineering of Dopamine Neurons,
Advances in Experimental Medicine and Biology, Volume 651

Axon Guidance in the Dopamine System

Asheeta A. Prasad and R. Jeroen Pasterkamp*

Abstract

Meso-diencephalic dopamine neurons (mdDA) neurons are located in the retrorubral field (RRF), substantia nigra pars compacta (SNc) and ventral tegmental area (VTA) and give rise to prominent ascending axon projections. These so-called mesotelencephalic projections are organized into three main pathways: the mesostriatal, mesocortical and mesolimbic pathways. Mesotelencephalic pathways in the adult nervous system have been studied in much detail as a result of their important physiological functions and their implication in psychiatric, neurological and neurodegenerative disease. In comparison, relatively little is known about the formation of these projection systems during embryonic and postnatal development. However, understanding the formation of mdDA neurons and their projections is essential for the design of effective therapies for mdDA neuron-associated neurological and neurodegenerative disorders. Here we summarize our current knowledge of the ontogeny of mdDA axon projections in subsystems of the developing rodent central nervous system (CNS) and discuss the cellular and molecular mechanisms that mediate mdDA axon guidance in these CNS regions.

Introduction

Meso-diencephalic dopamine neurons (mdDA) neurons acquired their name from their origin within the mes- and diencephalon and from their characteristic expression of the neurotransmitter dopamine.[1,2] Anatomically and functionally distinct groups of mdDA neurons have been identified in the retrorubral field (RRF or A8), the substantia nigra pars compacta (SNc or A9) and the ventral tegmental area (VTA or A10).[3-5] MdDA neurons give rise to prominent ascending axon projections. These so-called mesotelencephalic projections are organized into three main pathways: the mesostriatal, mesocortical and mesolimbic pathways.[1] SNc neurons project axons to the dorsal striatum forming the mesostriatal pathway. The mesostriatal pathway is involved in the coordination of voluntary movement, which is dramatically demonstrated by the symptoms of Parkinson's disease (PD). In PD patients, mdDA neurons in the SNc degenerate leading to impaired motor control and even complete loss of movement in extreme cases.[6] Distinct from SNc projections, VTA and RRF axons prominently innervate the ventromedial striatum and prefrontal cortex (PFC), contributing to the mesolimbic and mesocortical pathways, respectively. Mesocorticolimbic projections are involved in the regulation of emotions and reward and defects in mesocorticolimbic connectivity have been implicated in addictive behavior, depression and schizophrenia.[7-10] The anatomical, functional and molecular properties of mesotelencephalic pathways in the adult nervous system have been studied intensely as a result of their important physiological functions and

*Corresponding Author: R. Jeroen Pasterkamp—Department of Neuroscience and Pharmacology, Rudolf Magnus Institute of Neuroscience, University Medical Center Utrecht, Universiteitsweg 100, 3584 CG, Utrecht, The Netherlands. Email: r.j.pasterkamp@umcutrecht.nl

Development and Engineering of Dopamine Neurons, edited by RJ Pasterkamp, MP Smidt and JPH Burbach. ©2008 Landes Bioscience and Springer Science+Business Media.

their implication in human disease. In comparison, relatively little is known about the formation of these projection systems during embryonic and postnatal development.[11] Here we summarize our current understanding of the development of mdDA projections in rodents, which have been studied in most detail. In each of the sections outlined below, a brief description of the ontogeny of mDA projections in a specific part of the developing central nervous system (CNS) is followed by a summary of our present knowledge of the cellular and molecular mechanisms that regulate mdDA axon guidance in these CNS regions.

Mesencephalon

In rodents, mdDA neurons are born around E10.5 (mouse) or E12 (rat) and begin to extend neurites at E11.5 (mouse) or E13 (rat).[2] Initially, these neurites follow a dorsal trajectory within the mesencephalon but then deflect rostrally towards the diencephalon (Fig. 1A, B).[12-14] Chemotropic factors in the floor plate, caudal brain stem and dorsal mesencephalon (DM) may contribute to this reorientation of mdDA axons (Fig. 2A). Tissue culture studies show that repulsive cues for mdDA axons emanate from the floor plate and caudal brain stem region in vitro.[12,15,16] Interestingly, the secreted axon repellent Slit3 is expressed at high levels in the caudal mesencephalon (Fig. 2B).[17] MdDA neurons express the Slit receptors Robo1 and Robo2[17,18] and Slit3 robustly repels embryonic mdDA axons in collagen matrix assays in vitro (Table 1).[19] It is therefore tempting to speculate

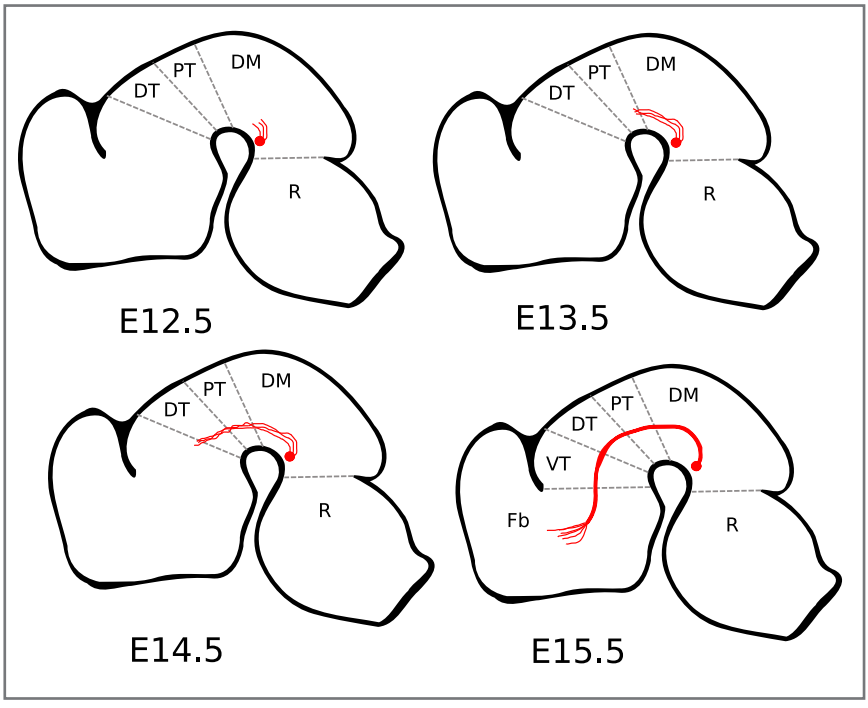

Figure 1. The ontogeny of mesodiencephalic dopamine (mdDA) axon projections during early to midembryonic development. Initially mdDA axons follow a dorsal trajectory (A) but then deflect rostrally towards the diencephalon (B). (C) In the diencephalon, mdDA axons course ventrally and then reorient into a rostral direction at the border of the diencephalon and telencephalon. (D) Around E14, mdDA axons reach an area ventrolateral to the developing striatum. Over the next few days the number of axons in this region increases without advancing notably beyond or into the overlying striatum. Around E17, the first mdDA axons begin to enter the developing striatum (not shown).

that the caudal brainstem region secretes Slit3 to repel mdDA axons towards the diencephalon. An elegant study by Nakamura and colleagues further reveals a rostrocaudal or caudorostral gradient of short-range directional cues in the DM that may also contribute to the stereotypic rostral trajectory of mdDA axons in the mesencephalon (Fig. 2A).[14] The DM region expresses another member of the Slit family, Slit1 (Fig. 2B).[17] Like Slit3, Slit1 is a potent repellent for mdDA axons acting through Robo receptors (Table 1).[19] Although Slits are secreted molecules, they are known to form tight interactions with the extracellular matrix.[20] Therefore, Slit1 may serve as a short-range repulsive guidance cue for mdDA axons in the DM. It should be noted, however, that in addition to Slit1 other axon repellents are expressed in the DM (e.g., Sema3F) that may impose the rostral trajectory of mdDA axons (Fig. 2B).[13,21] Furthermore, the results of Nakamura et al do not exclude the possibility that a gradient of chemoattractive molecules enforces the reorientation of mdDA axons in the DM. Supportively, mRNA expression data suggest that Sema3C labels the future trajectory of mdDA axons in the mesencephalon. Sema3C is a strong chemoattractant for embryonic mdDA fibers (Table 1) and may attract these axons towards the rostral part of the mesencephalon in vivo (Fig. 2B).[13] Future studies employing genetically modified mice and immunohistochemical stains, to establish Slit and Sema3 protein expression in the DM, are needed to determine if the combined actions of Slits and Sema3s dictate to the stereotypic trajectory of mdDA axons in the mesencephalon.

Diencephalon

In the diencephalon, mdDA axons course ventrally and then reorient into a rostral direction at the border of the diencephalon and telencephalon (Fig. 1C).[12-14,22] To identify factors that control the trajectory of mdDA projections in the diencephalon, explants derived from different parts of the embryonic diencephalon were cocultured with mdDA neuron-containing mesencephalon explants.[13] Explants from the rostral pretectum (PT) were found to exert growth-promoting and attractive effects on mdDA axons, whereas dorsal and ventral thalamus tissues did not influence mdDA axon growth and guidance (Fig. 2A).[13] The rostral part of the PT expresses two secreted semaphorins, Sema3C and Sema3F, with opposing effects on mdDA directional growth in vitro. While Sema3C attracts mdDA axons in culture, Sema3F strongly repels these axons (Table 1). A subset of mdDA neurons expresses neuropilin (Npn)-1 and/or Npn-2, obligatory components of Sema3 receptors and the axon attractive effect of the rostral PT can be neutralized by blocking Npn-1 and Npn-2 in vitro.[13] Overall, these results suggest that axonal attractant Sema3C is (partly) responsible for the attractive effect of the rostral PT on mdDA axons (Fig. 2B). The observation that the PT also contains the axon repellent Sema3F raises the question why the PT has a net attractive and not repulsive effect. A possible explanation for this observation is that the attractive effect of Sema3C masks Sema3F repulsion because of higher expression levels of the former or through mechanisms involving competitive agonism, as has been shown for Sema3A, Sema3B and Sema3C.[23] Future analyses of *Npn* or *Sema3*-deficient mice will help to establish the role of Sema3C and other Sema3s in mdDA pathfinding in the diencephalon.

Medial Forebrain Bundle

Within the diencephalon and telencephalon mdDA axons diverge into two tightly fasciculated axonal tracts, forming the medial forebrain bundles (MFBs). The molecular cues that control the fasciculation of mdDA axons into two ipsilateral MFBs are unknown but the MFB region itself is known to exert potent chemotropic effects on mdDA axons. Coculture studies indicate that MFB explants derived from E12 and E15 rat embryos have an attractive effect on mdDA axons.[12] This suggests that the MFB region produces chemoattractant molecules that guide mdDA axons toward the rostral telencephalon. Remarkably, E19 MFB explants no longer attract (or repel) mdDA axons.[12] This specific regulation of the chemotropic properties of the MFB region may allow mdDA axons to exit the MFB at mid-to-late embryonic stages and to proceed towards their synaptic targets. Similar molecular mechanisms have been reported for axonal projections in other regions of the CNS including the spinal cord.[24]

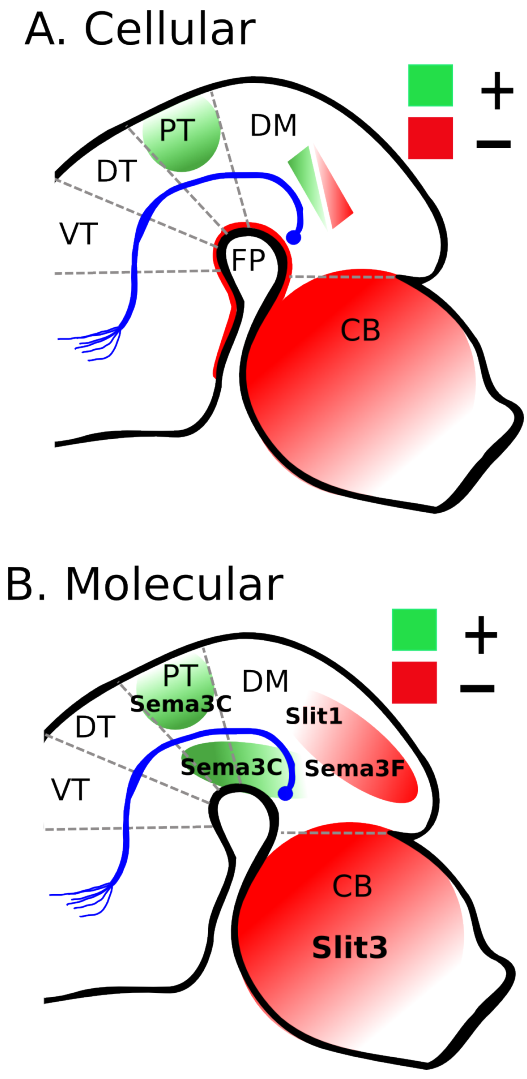

Figure 2. Axon guidance of mesodiencephalic dopamine (mdDA) neurons in the embryonic mesencephalon and diencephalon. A) Schematic representation indicating the different brain regions that provide chemotropic signals for embryonic mdDA axons as determined by in vitro experiments. Regions displaying chemorepulsive (-) factors and chemoattractive (+) effects are indicated. The floor plate (FP) and caudal brainstem (CB) regions produce chemorepellent molecules that may help to reorient mdDA axons rostrally. This rostral path is further enforced by a rostrocaudal or caudorostral gradient of chemoattractants or chemorepellents, respectively, in the dorsal mesencephalon (DM). The rostral part of the pretectum (PT) attracts mdDA axons in vitro. B) Schematic representation indicating the axon guidance molecules that have been proposed to mediate guidance events in the mesencephalon and diencephalon. Slit3 is expressed in the CB and may function to repel mdDA axons rostrally. This reorientation into a rostral direction may furthermore be controlled by a caudorostral gradient of the axon repellents Slit1 and/or Sema3F. Sema3C is expressed along the mdDA trajectory in the mesencephalon and in the PT where it mediates chemoattractive responses. DT, dorsal thalamus; VT, ventral thalamus.

Table 1. Axon guidance molecules for mesodiencephalic dopamine (mdDA) neurons

AGM	In Vitro Effect on mdDA Axons	Receptor In Vitro	KO Mouse Analysis of mdDA Axon Pathways	References
EphrinB2	Axon growth inhibition	nd	nd	Yue et al, 1999
	Neuronal cell death	nd	nd	Yue et al, 1999
Netrin-1	Axon attraction	DCC	nd	Lin et al, 2005
	Axon growth promotion	DCC	nd	Lin et al, 2005
Sema3A	Axon growth promotion	Npn-1	nd	Hernandez-Montiel et al, 2008
Sema3C	Axon attraction	Npn-1/Npn-2	nd	Hernandez-Montiel et al, 2008
Sema3F	Axon repulsion	Npn-2	nd	Hernandez-Montiel et al, 2008
Slit1	Axon repulsion	Robo	no defects reported *	Bagri et al, 2002; Lin and Isacson, 2006
Slit2	Axon repulsion	Robo	abnormal ventral trajectory*	Bagri et al, 2002; Lin and Isacson, 2005, 2006
	Axon branching	Robo	-	Lin et al, 2005
Slit3	Axon repulsion	Robo	nd	Lin and Isacson, 2006

*In *Slit1; Slit2* double mutant mice the MFB is split, mdDA axons descend aberrantly into the hypothalamus and cross the midline. Abbreviations: DCC: deleted in corectal cancer; nd: not determined; Npn: neuropilin; Robo: roundabout.

Brain regions flanking the presumptive trajectory of the MFB such as the thalamus and hypothalamus have been proposed to dictate the characteristic ventrolateral position of the MFBs in the telencephalon. Although thalamic explants have no long-range chemotropic effects on mdDA axons in vitro, mdDA axons do not enter thalamic explants.[12] This observation hints at the presence of contact-dependent inhibitors of mdDA axon outgrowth in the embryonic thalamus. The idea that medial brain structures contribute to the positioning of the MFBs is further supported by the disorganization of the caudal hypothalamus and concomitant aberrant midline crossing of MFB fibers in *Nkx2.1* mutant mice.[25,26] Based on the reduced expression of Sema3A in the caudal hypothalamus of *Nkx2.1* mutant mice it was postulated that Sema3A acts as a midline repellent for mdDA axons.[25] However, the finding that Sema3A functions a chemotrophic rather than a chemotropic cue for mdDA axons in vitro contrasts this idea.[13] It should be noted, however, that individual Sema3s are bifunctional and can exert repulsive, attractive or axon growth promoting effects depending on the biological context in which they are encountered.[27] Since the biological context of cultured neurons is likely to be different from in vivo conditions further work is needed to study the role of Sema3A in vivo. Alternative explanations for the abnormal crossing of MFB fibers in the absence of Nkx2.1 include the loss of a physical instead of a molecular barrier for axon growth and the decreased expression of repulsive cues other than Sema3A such as Slit2. Slit2 repels mdDA axons in vitro (Table 1)[18] and mdDA axons are displaced ventrally as they course through the diencephalon of *Slit2* mutant mice.[28] In addition, in *Slit1; Slit2* double mutants, the MFB splits into two components and mdDA axons descend ventrally into the hypothalamus towards the midline. Furthermore, many fibers abnormally cross the midline at the level of the basal telencephalon in the absence of Slit1 and Slit2.[28] The observation that *Nkx2.1* mutant mice display changes in *Slit* expression[26] and wiring defects resembling those observed in *Slit* mutants suggests that abnormal Slit function may underlie the pathfinding errors observed in mice lacking Nkx2.1. However, mdDA pathways are more severely and in part also differently affected in *Nkx2.1* as compared to *Slit* mutants. In addition, many mdDA axons project normally in *Slit1; Slit2* double mutants. Thus multiple distinct guidance cues are needed for the proper formation of mdDA pathways.

Striatum

Around E14 in rat, mdDA axons in the MFB reach and invade the region ventrolateral to the developing ganglionic eminence (GE)/striatum. Over the next few days the number of axons in this region increases without advancing into a rostral or dorsal direction (Fig. 1D). Around E17, mdDA axon bundles begin to enter the developing striatum coincident with the emergence of a chemoattractive activity from the striatal region. Intriguingly, both late embryonic and postnatal but not early embryonic striatal explants attract mdDA explants in vitro.[12,13,22,29-31] This suggests that the aforementioned 'waiting period' for mdDA axons may be a consequence of the lack of chemoattraction by the early embryonic striatum. The molecules that mediate these chemoattractive effects are unknown. Interestingly, Sema3A is expressed by the embryonic striatum at the stage when mdDA axons enter the striatum.[13,32,33] However, function-blocking antibodies against the Sema3A receptor component Npn-1 do not neutralize the attractive effect of striatal explants on mdDA axons.[13] Another brain structure that may help to enforce the 'waiting period' for mdDA axons ventrolateral to the striatum is the neocortex. Cortical explants exert a repulsive effect on mdDA axons in vitro[12] and molecular cues emanating from the cortex could inhibit the rostral and dorsal progression of mdDA fibers.

From E17 onwards, rat mdDA axons penetrate the developing striatum. From their initial ventrolateral position mdDA, axons start to extend into dorsal, medial, lateral and rostral directions to establish the topographic connections that are found in the adult. In the adult, mdDA neurons in the SNc densely innervate the dorsal striatum, while VTA neurons predominantly target the ventral striatum.[1] The formation of these topographic connections is controlled by specific axonal pruning events during late embryonic and early postnatal development.[34] In contrast to the adult situation, axon collaterals from mdDA neurons in the embryonic VTA and SNc (E15, E17) innervate both

the dorsal and ventral striatum. Topographic specificity is achieved during late embryonic and early postnatal development through the selective elimination of axon collaterals from the SNc and VTA targeting the ventral and dorsal striatum, respectively.[34] Although the molecular basis of these axonal pruning events remains to be established, Eph and ephrins have been implicated in the formation of topographic connections between the dopaminergic mesencephalon and the striatum.[35] EphB1 is expressed at high levels in the SN, but only weakly in the VTA. In the embryonic striatum, the EphB1 ligand ephrinB2 is strongly expressed in the ventromedial striatum (targeted by VTA axons) but only weakly in the dorsolateral striatum (targeted by SNc axons). Thus, SNc neurons with high levels of EphB1 project to the dorsolateral striatum which weakly expresses ephrinB2. In contrast, VTA neurons, which express low levels of EphB1, innervate the ventromedial striatum where high levels of ephrinB2 are found. Tissue culture studies show that ephrinB2 inhibits the growth of EphB1-positive SNc neurons in vitro.[35] Furthermore, ephrins can induce axonal degeneration of cultured hippocampal neurons.[36] Overall, these data suggest that ephrinB2 may regulate the formation of topographic mdDA projections by acting as an axon guidance and/or pruning factor for SN collaterals. It should be noted, however, that analysis of *EphB1* mutant mice is incoherent to the idea that EphB1 functions in the formation of mesostriatal connections.[37] This suggests that in SNc neurons Eph receptors other than EphB1 may be involved in detecting ephrinB2 expression in the striatum. Genetic manipulation of another Eph, EphA5, results in prominent defects in mesostriatal projections. Mice overexpressing an extracellular fragment of EphA5 (EphA5-Fc), known to antagonize ephrinA signaling,[38] display a reduction in the number of mesostriatal projections in adulthood.[39,40] The observation that neuronal survival is unaffected in EphA5-Fc mice supports the idea that EphA5-ephrinA signaling is required for the maintenance and/or formation of mesostriatal projections.

Cortex

The prefrontal cortex (PFC) receives a dense dopaminergic innervation. The first mdDA axons reach the rat PFC around E15 and at first remain confined to the subplate (SP). Over the next few days, the number mdDA axons in the SP increases but no penetration of the overlying cortical plate can be observed until E17-E18. Thus, similar to above mentioned 'waiting period' for mesostriatal projections, mesocortical axons stall for several days before entering more superficial layers of the developing cortex.[41] The molecular cues that regulate the spatiotemporal innervation of the PFC by mdDA axons remain to be identified. Following their initial penetration of the CP, mdDA axons continue to establish layer-specific and topographic connections with the PFC.[41,42] Remarkably and as stated above, cortical tissue strongly repels mdDA axons in vitro.[12] However, the cortex is a large and heterogeneous structure and the origin of the cortical explants used in this study was not specified. Other work, employing cocultures of dissociated mesencephalic and cortical cells, shows that neurons from different cortical regions exert differential effects on the maturation of mdDA neurons.[43] These results indicate that characterization of chemotropic influences exerted by (non)target regions in the cortex on mdDA axons will require microdissection and testing of cortical subregions.

Axon Guidance Molecules and Disease

Studying the cellular and molecular basis of mdDA neural connectivity allows understanding the factors underlying disease onset, progression and furthers the development of new therapeutic strategies, particularly within regenerative medicine. For example, insight into the molecular control of mesotelencephalic pathway formation and maintenance may help to repair the mdDA system of PD patients and could also provide insight into the onset and progression of this disorder. PD was originally identified by James Parkinson in 1817 on the basis of severe motor dysfunction. Succeeding analysis of postmortem tissue revealed a characteristic loss of SN mdDA neurons. Current therapies for treating PD aim to restore cerebral dopamine levels by administrating levodopa, a prodrug that is converted to dopamine by the enzyme tyrosine hydroxlyase in dopaminergic neurons. However, due to the progressive neurodegenerative nature of PD, the effect of

levoda is only transient.[6] Long-term goals of recovery strive to maintain stable dopamine levels by transplanting dopamine-producing cells into the brain of PD patients.[44] The pitfalls of this approach are the quantities of high-quality cells required for transplantation and the inability of many of the grafted neurons to establish functional neural connections. The vital role of axon guidance molecules in cell replacement thereapies is well recognized. A recent study shows that ES cell-derived mdDA neurons are responsive to guidance cues such as Slits and netrin-1.[19] Furthermore, the axons of transplanted neurons can be guided in vivo by ectopic expression of guidance cues.[45,46] Therefore, axon guidance molecules may serve to assist grafted mdDA neurons in making successful new connections and in preventing them from forming the inappropriate connections believed to underlie some of the side effects of cell replacement strategies. In addition to the role of axon guidance molecules in guiding newly formed projections of transplanted mdDA neurons, several studies have shown significant differences in axon guidance gene expression between control and PD patients or PD mouse models.[47-50] Furthermore, genomic pathway approaches identify polymorphisms in the axon guidance pathways of PD patients.[51] Although further work is needed to establish the role of abnormal axon guidance cue function in the pathophysiology of PD, changes in the expression of axon guidance molecules could lead to altered patterns of neuronal connectivity in the mdDA system and as a consequence to neuronal dysfunction and loss.

The mesocorticolimbic system is involved in reward seeking behavior and is affected during drug addiction.[9,10] Drug induced effects can be become perpetual where development and maintenance of addiction cause changes in neural morphology and synaptic activity.[52] Interestingly, the expression of axon guidance molecules is modulated during the development of drug addiction.[35,39,53,54] Cocaine induces significant changes in gene expression of several members of semaphorin and Eph/ephrin families in the NAc and VTA region.[53] Additionally, disruption of EphA/ephrin-A signaling induces changes in the behavioral response to psychostimulants.[40] It is therefore tempting to speculate that drug-induced changes in the expression of axon guidance molecules contribute to the structural adaptations that underlie the long-term effects of prolonged drug exposure.

Changes in dopaminergic neurotransmission have also been implicated in neurological diseases such as depression and schizophrenia.[7,8,55,56] Although it remains to be determined whether structural changes in dopaminergic connections underlie these alterations in neurotransmission, genetic studies link axon guidance cues to several of these disorders. Whether or not dysregulation of axon guidance molecule expression is coupled with these disorders remains to be investigated.

Conclusions and Future Directions

Mesotelencephalic projections mediate a wide range of physiological functions and are affected in various neurological, psychiatric and neurodegenerative disorders. It is well recognized that insight into the mechanisms that control the formation and maintenance of mesotelencephalic projections is essential for understanding and treatments of perturbed mdDA connectivity. Unfortunately, relatively little is known about the molecular signals that control the wiring of the mdDA system. Work during the past few years has identified several different axon guidance molecules that may control the formation of mesotelencephalic projections. However, most of our current knowledge of mdDA axon guidance derives from gene expression analysis and in vitro studies and validation in in vivo models will be required to establish how axon guidance cues function in concert to establish functional mesotelencephalic connections.

Acknowledgements

The authors thank Marten Smidt en Peter Burbach for critically reading the manuscript and members of the Pasterkamp lab for helpful discussions. Work in the authors' laboratory is supported by grants from Netherlands Organization of Scientific Research, Dutch Brain Foundation, the International Parkinson Foundation, the Human Frontier Science Program and ABC Genomics Center Utrecht (to RJP). RJP is a NARSAD Henry and William Test Investigator.

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Chapter 3

Wnt/Planar Cell Polarity Signaling Controls the Anterior–Posterior Organization of Monoaminergic Axons in the Brainstem

Asheeta A. Prasad,[2*] Ali G. Fenstermaker,[1*] Ahmad Bechara,[1] Youri Adolfs,[2] Fadel Tissir,[3] Andre Goffinet,[3] Yimin Zou,[1] and R. Jeroen Pasterkamp[2]

*Equal contribution. [1]Neurobiology Section, Biological Science Division, University of California, San Diego, La Jolla, California 92093, [2]Department of Neuroscience and Pharmacology, Rudolf Magnus Institute of Neuroscience, University Medical Center Utrecht, 3584 CG Utrecht, The Netherlands, and [3]Universit le Catholique de Louvain, Institute of Neuroscience, B-1200 Brussels, Belgium

Journal of Neuroscience, 2010

The Journal of Neuroscience, November 24, 2010 • 30(47):16053–16064 • **16053**

Development/Plasticity/Repair

Wnt/Planar Cell Polarity Signaling Controls the Anterior–Posterior Organization of Monoaminergic Axons in the Brainstem

Ali G. Fenstermaker,[1]* Asheeta A. Prasad,[2]* Ahmad Bechara,[1] Youri Adolfs,[2] Fadel Tissir,[3] Andre Goffinet,[3] Yimin Zou,[1] and R. Jeroen Pasterkamp[2]

[1]Neurobiology Section, Biological Science Division, University of California, San Diego, La Jolla, California 92093, [2]Department of Neuroscience and Pharmacology, Rudolf Magnus Institute of Neuroscience, University Medical Center Utrecht, 3584 CG Utrecht, The Netherlands, and [3]Université Catholique de Louvain, Institute of Neuroscience, B-1200 Brussels, Belgium

Monoaminergic neurons [serotonergic (5-HT) and dopaminergic (mdDA)] in the brainstem project axons along the anterior–posterior axis. Despite their important physiological functions and implication in disease, the molecular mechanisms that dictate the formation of these projections along the anterior–posterior axis remain unknown. Here we reveal a novel requirement for Wnt/planar cell polarity signaling in the anterior–posterior organization of the monoaminergic system. We find that 5-HT and mdDA axons express the core planar cell polarity components Frizzled3, Celsr3, and Vangl2. In addition, monoaminergic projections show anterior–posterior guidance defects in *Frizzled3*, *Celsr3*, and *Vangl2* mutant mice. The only known ligands for planar cell polarity signaling are Wnt proteins. In culture, Wnt5a attracts 5-HT but repels mdDA axons, and Wnt7b attracts mdDA axons. However, mdDA axons from *Frizzled3* mutant mice are unresponsive to Wnt5a and Wnt7b. Both Wnts are expressed in gradients along the anterior–posterior axis, consistent with their role as directional cues. Finally, *Wnt5a* mutants show transient anterior–posterior guidance defects in mdDA projections. Furthermore, we observe during development that the cell bodies of migrating descending 5-HT neurons eventually reorient along the direction of their axons. In *Frizzled3* mutants, many 5-HT and mdDA neuron cell bodies are oriented abnormally along the direction of their aberrant axon projections. Overall, our data suggest that Wnt/planar cell polarity signaling may be a global anterior–posterior guidance mechanism that controls axonal and cellular organization beyond the spinal cord.

Introduction

Axon guidance cues control the direction of growth cone navigation along the major anatomical axes [anterior–posterior (A–P), dorsal–ventral, and inferior–superior] during neural circuit development. Wnt family proteins are evolutionary conserved A–P guidance cues (Zou, 2006; Zou and Lyuksyutova, 2007). In the vertebrate spinal cord, Wnts provide directional cues for both ascending sensory axons and descending motor axons (Lyuksyutova et al., 2003; Liu et al., 2005; Wolf et al., 2008). In contrast, the A–P axon guidance cues that function in the vertebrate CNS more rostral to the spinal cord remain essentially unknown.

Monoaminergic neurons [serotonergic (5-HT) and dopaminergic (mdDA)] in the brainstem control many aspects of nervous system functioning, and their malfunctions are involved in the pathogenesis of a large number of nervous system diseases, such as depression, autism, and Parkinson's disease (Pardo and Eberhart, 2007; Van den Heuvel and Pasterkamp, 2008). These neurons are organized in discrete nuclei in the midbrain (mdDA; A9, A10) and hindbrain (5-HT; B1–B9) and project axons along the A–P axis to reach their final targets in the brain and spinal cord (Rubenstein, 1998; Goridis and Rohrer, 2002; Cordes, 2005; Smidt and Burbach, 2007; Van den Heuvel and Pasterkamp, 2008). The A–P projections of 5-HT and mdDA neurons are established during midgestation and are essential for appropriate monoaminergic circuit formation during subsequent developmental stages. Surprisingly little is known about the genetic control of monoaminergic pathway development, particularly how monoaminergic projections are initially established along the A–P axis.

One of the Wnt signaling pathways, the planar cell polarity (PCP) pathway, is involved in tissue morphogenesis and directed cell migration (Wang and Nathans, 2007; Zallen, 2007; Goodrich, 2008; Simons and Mlodzik, 2008). Core PCP components include Frizzled, Flamingo (Celsrs in vertebrates), Van Gogh,

Received Aug. 28, 2010; accepted Oct. 2, 2010.

This work is supported by National Institutes of Health Grant R01 NS047484 (Y.Z.) and by the Netherlands Organization for Health Research and Development (ZonMW-VIDI and ZonMW-TOP), the International Parkinson Foundation, the Human Frontier Science Program (Career Development Awards), and the European Union (mdDA-Neurodev, FP7/2007-2011, Grant 222999) (R.J.P.). This study was partly performed within the framework of Dutch Top Institute Pharma Project T5-207 (R.J.P.). We are grateful to Tony Wynshaw Boris for sharing the *Looptail* mice, Steven Stacker for providing the *Ryk* mutant mice, and Francesca Morello and Katsumi Fumoto for help with immunohistochemistry and confocal microscopy. We thank Rejji Kuruvilla and Jeremy Nathans for generously providing the *Fzd3* mutant mice.

*A.G.F. and A.A.P. contributed equally to this work.

Correspondence should be addressed to either of the following: Yimin Zou, Neurobiology Section, Biological Science Division, University of California, San Diego, La Jolla, CA 92093, E-mail: yzou@ucsd.edu; or Jeroen Pasterkamp, Department of Neuroscience and Pharmacology, Rudolf Magnus Institute of Neuroscience, University Medical Center Utrecht, 3584 CG Utrecht, The Netherlands, E-mail: r.j.pasterkamp@umcutrecht.nl.

DOI:10.1523/JNEUROSCI.4508-10.2010

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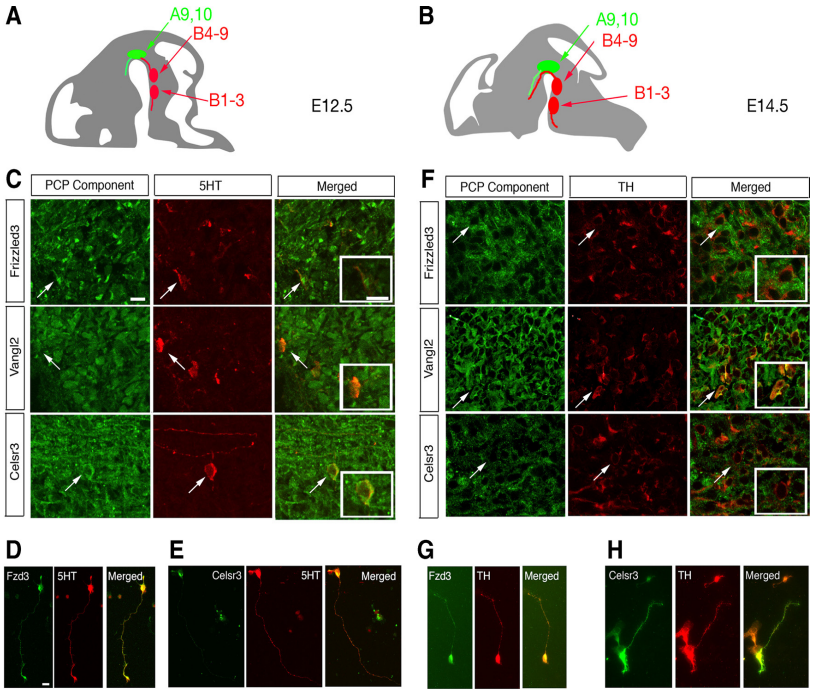

Figure 1. PCP signaling components are expressed in 5-HT- and TH-positive neurons during midbrain and hindbrain development. Schematics in ***A*** and ***B*** indicate the anatomical location of the descending and ascending serotonin systems (B1–B3 and B4–B9; red) and the mesodiencephalic dopamine system (A9, A10; green) in mouse at E12.5 and E14.5, respectively. ***C***, Immunohistochemistry for 5-HT in red and PCP receptors in green in sagittal sections through the embryonic hindbrain at E12.5. Frizzled3, Vangl2, and Celsr3 all colocalize with 5-HT-positive neurons and fibers. Insets depict higher-magnification images of cells indicated by the arrow. ***D***, Confocal images showing immunohistochemistry for TH in red and PCP receptors in green in coronal sections through the E14.5 midbrain. Frizzled3, Vangl2, and Celsr3 all colocalize with TH-positive neurons and fibers. Insets depict higher-magnification images of cells indicated by the arrow. ***D***, ***E***, 5-HT-positive dissociated neurons coimmunostained with 5-HT in red and Fzd3 (***D***) or Celsr3 (***E***) in green. ***G***, ***H***, TH-positive dissociated neurons coimmunostained with TH in red and Fzd3 (***G***) or Celsr3 (***H***) in green. Scale bars, 20 μm.

Disheveled, Prickle, and Diego. In this study, we found that Wnt/PCP signaling is essential for the A–P organization of 5-HT and mdDA axons in the brainstem. Both 5-HT and mdDA neurons expressed core PCP components and showed A–P guidance defects in *Frizzled3* (*Fzd3*), *Celsr3*, and *Vangl2* [*Looptail* (*Lp*)] mutant mice. In addition, *Wnt5a* mutants showed transient A–P guidance defects for mdDA axons. *Wnt5a* and *Wnt7b* were expressed in gradients along the A–P axis in the brainstem, consistent with their role as directional cues. In culture, Wnt5a attracted 5-HT axons but repelled mdDA axons, whereas Wnt7b attracted mdDA axons. In contrast, dopaminergic neurons derived from *Fzd3* mutant mice did not respond to Wnt5a or Wnt7b. Analyses of wild-type or *Fzd3* mutant mice further revealed that cell bodies of descending 5-HT neurons are initially oriented mediolaterally and gradually shift to orient along the A–P axis following the direction of their axons and that proper Wnt/PCP signaling is essential for this A–P cellular organization of monoaminergic nuclei. Together, these results define novel and crucial roles for Wnt/PCP signaling in establishing the A–P axonal and cellular organization of monoaminergic systems. Also, these findings indicate that A–P axon guidance functions of different PCP proteins are not limited to the spinal cord but function more globally in the vertebrate CNS.

Materials and Methods

Animals. All animal use and care were in accordance with institutional guidelines. CD-1 and C57BL/6 mice were obtained from Charles River. *Fzd3* mutant mice were obtained from Jeremy Nathans [Johns Hopkins University School of Medicine, Baltimore, MD (Wang et al., 2002)], *Looptail* mice were from Tony Wynshaw Boris [University of California, San Francisco, San Francisco, CA (Montcouquiol et al., 2006)], and *Ryk* mutant mice were from Steven Stacker [Ludwig Institute for Cancer Research, Melbourne, VIC, Australia (Halford et al., 2000)]. *Celsr3* mutant mice have been described previously (Tissir et al., 2005). *Wnt5a* mutant mice were purchased from The Jackson Laboratory. Timed-pregnant mice were killed by means of cervical dislocation. The morning on which a vaginal plug was detected was considered embryonic day 0.5 (E0.5). For *in situ* hybridization and immunohistochemistry, E11.5–E17.5 brains were fixed in 4% paraformaldehyde (PFA) for 30 min to 1 h, followed by immersion in 30% sucrose. Brains were frozen on powdered dry ice, cryosectioned, mounted on Superfrost Plus slides (Thermo Fisher Scientific), air dried, and stored desiccated at −80°C.

In situ *hybridization and immunohistochemistry.* Nonradioactive *in situ* hybridization on cryosections (14 μm) was performed as described previously (Pasterkamp et al., 2007). Specific digoxigenin-labeled *in situ* probes for the mouse Wnt family were as described (Liu et al., 2005). Whole-mount *in situ* hybridization was preformed as described previously (Henrique et al., 1995).

Cryosections (14 μm) were stained immunohistochemically as described previously (Kolk et al., 2009). Sections were counterstained with

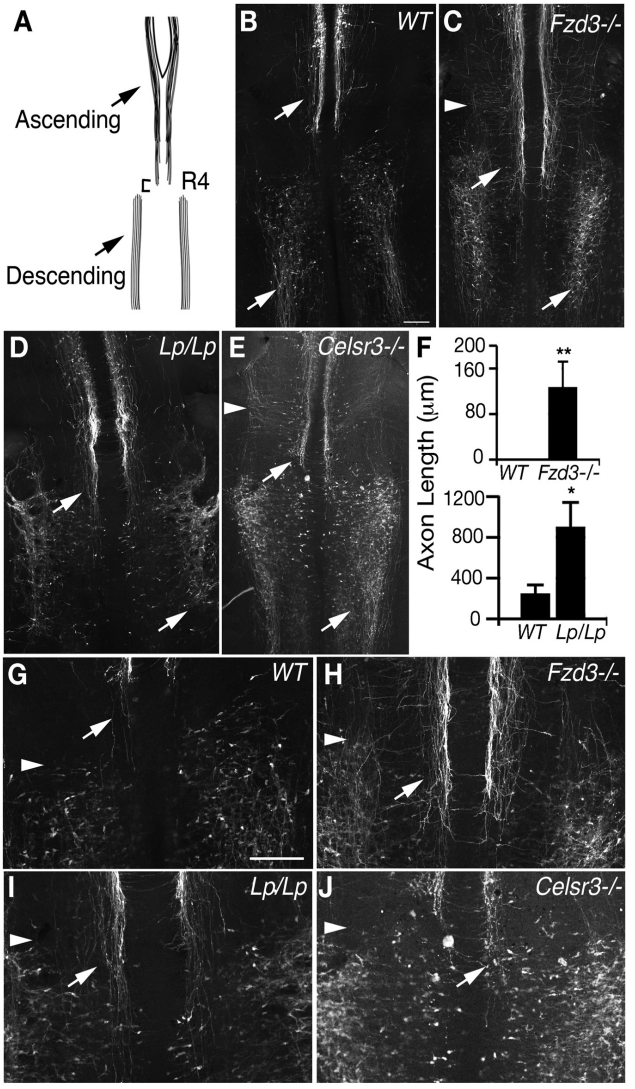

Figure 2. PCP signaling is required for axonal pathfinding of hindbrain serotonergic neurons. ***A***, Schematic representation of the developing 5-HT system at E12.5. ***B–E***, Whole-mount immunostaining against 5-HT in the hindbrain. At E12.5, ascending 5-HT neurons in *Fzd3*$^{-/-}$ (***C***), *Lp/Lp* (***D***), and *Celsr3*$^{-/-}$ (***E***) mice all display axon misprojections into R4 (see top arrows). *Fzd3*$^{-/-}$ and *Celsr3*$^{-/-}$ also display laterally projecting axons within the ascending population (arrowheads in ***C***, ***E***). In addition, *Fzd3*$^{-/-}$ (***C***) and *Lp/Lp* (***D***) mice display aberrant anteriorly projecting 5-HT projections in the descending population and a marked reduction of proper descending axons (bottom arrows). ***F***, Length of axon invasion into R4 in the *Fzd3*$^{-/-}$ and *Lp/Lp* mouse. ***G–J***, Higher magnification of premature R4 invasion. Arrowheads indicate the start of the descending population, and the arrow label axons from the ascending population. Scale bar, 250 μm. WT, Wild type. $^{**}p < 0.005$, $^{*}p < 0.03$.

fluorescent Nissl (1:500; Neurotrace, Invitrogen) or 4′,6′-diamidino-2-phenylindole (DAPI) (Sigma), washed extensively, and embedded in Mowiol (Sigma). Staining was visualized using a Carl Zeiss Axioskop 2 microscope or by confocal laser-scanning microscopy (LSM510; Carl Zeiss). The following primary antibodies were used: rabbit anti-Frizzled3 (1:500; a gift from Jeremy Nathans), rabbit anti-Celsr3 (1:500; generated in the Zou laboratory), goat anti-Vangl2 (1:100; Santa Cruz Biotechnology), rat anti-5-HT (1:500; Accurate Chemicals), rabbit anti-tyrosine hydroxylase (TH) (1:1000; Pel-Freeze), and mouse anti-TH (1:500; Immunostar).

TH explant assays. Three-dimensional collagen matrix assays using substantia nigra (SN) and ventral tegmental area (VTA) explants were performed as described previously (Kolk et al., 2009). SN or VTA explants were rapidly dissected from E12.5–E14.5 C57BL/6 embryos or from wild-type or *Frizzled3* mutant embryos and embedded in close proximity to (~300 μm apart) aggregates of HEK293 cells transiently transfected with expression vectors for Wnt5a or Wnt7b, or mock-transfected as a control. Explants were cultured for 56–58 h, fixed, and coimmunostained using rabbit anti-TH (1:1000; Pel-Freeze) and mouse anti-βIII-tubulin (1:3000; Covance) antibodies to stain dopaminergic and all neurites emerging from the explants, respectively.

For quantification of explant assays, the length of the 20 longest TH-positive neurites was measured in both the proximal and distal quadrants of the explant cultures using OpenLab software (Improvision). The average value of the 20 neurites proximal and distal was used to determine the proximal/distal ratio (P/D ratio) per explant. Data were statistically analyzed by one-way ANOVA ($\alpha = 5\%$) and expressed as means ± SEM.

5-HT open-book explants. Hindbrains were dissected from E12.5 time pregnant CD-1 mice in L15 media and placed ventricular zone down onto a bed of collagen. Blue Sepharose beads previously coated overnight at 4°C in 100 ng/μl purified Wnt proteins were placed along the midline of the explants, and then the explant was covered in a top layer of collagen. Explants were incubated at 37°C for 24 h and fixed with 4% PFA overnight at 4°C. 5-HT-positive neurons were visualized using whole-mount immunostaining with rat anti-5-HT (1:300; Accurate Chemicals). Images were acquired by confocal microscopy, and *Z*-stacks were compressed and analyzed in NIH ImageJ.

For the quantification of hindbrain open-book cultures, we counted the number of 5-HT-positive axons emanating from the midline of the ascending population. The axons were grouped as growing anteriorly, laterally, or posteriorly. The number of axons growing anteriorly was graphed as a percentage of the total number of axons counted. More than 100 axons were counted for each explant (control, $n = 10$; Wnt4, $n = 6$; Wnt5a, $n = 10$; Wnt7a, $n = 9$). Data were statistically analyzed by one-tailed Student's *t* test and represented as ±SEM.

Quantification of premature axon invasion into rhombomere 4. For quantification of the axon guidance defect of the ascending 5-HT population, we measured the length of axons past the start of the descending neuron population (see Fig. 2*G–J*, arrowhead). Both *Fzd3* and *Lp* mice were compared with littermate controls because there were developmental differences among different genetic backgrounds (B6 mixed vs Sv129J mixed). This analysis was used to show that the mutants invaded the region below rhombomere 4 (R4) before their wild-type counterparts did. ($Fzd3^{+/+}$, $n = 6$, $Fzd3^{-/-}$, $n = 4$; $Lp^{+/+}$, $n = 4$; *Lp/Lp*, $n = 4$). Data were statistically analyzed by one-tailed Student's *t* test and represented as ±SEM.

Dissociated neuron cultures. To generate dissociated neuron cultures from the midbrain, the mdDA system was microdissected from E14.5 C57BL/6 wild-type embryos. Tissue from several different embryos was pooled and incubated in trypsin for 20 min at 37°C. Trypsin activity was inhibited with 20% fetal calf serum. Then, the cells were pelleted by mild centrifugation and resuspended in Neurobasal medium supplemented with B27, penicillin/streptomycin, and glutamine. A single-cell suspension was generated by putting the cells through a 70 μm filter after which cells were plated on poly-D-lysine- and laminin-coated glass coverslips in a 12-well plate for 2–3 d. Hindbrain neuron cultures were generated similar to dopaminergic neurons but with the following modifications. Hindbrains were dissected anterior to rhombomere 4 from E12.5 CD1 mouse embryos. Tissue from multiple embryos was pooled in incubated in 0.025% trypsin for 20 min at 37°C. The tissue was then triturated with fire polished glass pasture pipettes, and trypsin activity was inhibited by the addition of RPMI plus 5% horse serum. Cells were plated in Neurobasal media as above on poly-D-lysine- and laminin-coated glass coverslips and incubated for 2 d. All cells were fixed with 4% paraformaldehyde for 10 min at room temperature and immunostained with the following primary antibodies: rabbit anti-Frizzled3 (1:500; a gift from Jeremy Nathans), rabbit anti-Celsr3 (1:500), goat anti-Vangl2 (1:100; Santa Cruz Biotechnologies), rat anti-5-HT (1:500; Accurate Chemicals), rabbit anti-TH (1:1000; Pel-Freeze), and mouse anti-TH (1:500; Immunostar).

Quantification of cell body orientation. To assess and quantify mdDA cell body orientation defects in *Fzd3* mutant mice, sagittal sections from E12.5 $Fzd3^{-/-}$ embryos ($n = 3$) and littermate controls ($n = 3$) were immunostained with anti-TH antibodies and counterstained with DAPI. Images through the lateral and medial midbrain region were captured from these sections at identical medial to lateral locations using a Carl Zeiss Axioskop 2 microscope. Then, using Carl Zeiss Axiovision software, angles were determined between the lining of the mesencephalic flexure (baseline, represents 0°) and the trajectory of the initial axon segment just proximal to the TH-positive cell body. Fifty to 100 TH-positive neurons were measured per embryo at the lateral and medial levels, and angles were grouped (0/45°, 45/90°, etc.) and calculated as percentage of total.

To analyze the orientation of 5-HT cell bodies of the descending population, we imaged whole-mount immunostaining of 5-HT neurons using anti-5-HT antibodies from E12.5 $Fzd3^{-/-}$ ($n = 3$) and wild-type littermate controls ($n = 3$). *Z*-stack images were acquired by confocal microscopy and analyzed with NIH Image J. Cell body orientation was assessed by drawing a line vertically down to the center of the cell, followed by drawing an intersecting line through the center of the initial segment of the leading process. The angle of this intersection was measured in NIH Image J. Using this method, a completely horizontal cell would give an angle of 90°, whereas a completely vertical cell would yield an angle of 180°. More than 100 5-HT-positive cells were counted per embryo, and the angles were binned (0/45°, 45/90°, etc.) and graphed as percentage of total as for TH quantification. Data were statistically analyzed by one-tailed Student's *t* test and represented as ±SEM.

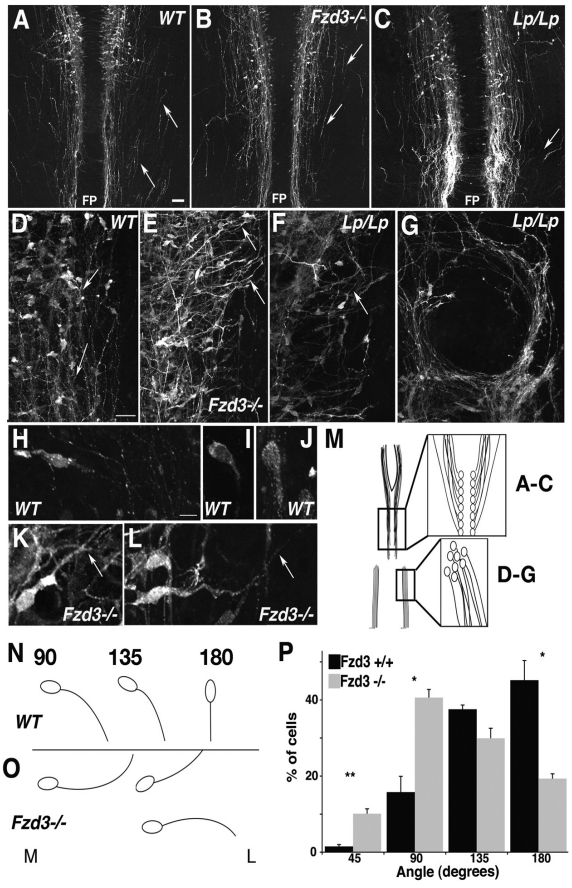

Figure 3. PCP mutant mice display specific axon guidance and cell body orientation defects in the 5-HT system. ***A–L***, Whole-mount 5-HT immunostaining. Ascending 5-HT populations are depicted in ***A–C***, and descending 5-HT axons are shown in ***D–L***. This is schematized in ***M***. ***A***, In E12.5 wild-type (WT) embryos, axons in the ascending 5-HT population emanate from the floor plate (FP) and strictly grow anterior toward the brain (arrows). In contrast, $Fzd3^{-/-}$ (***B***) or *Lp/Lp* (***C***) mice display ascending axons that grow laterally and posteriorly (arrows). *Lp/Lp* mice display many "wavy" axons characteristic of axons lacking appropriate guidance. ***D***, In wild-type mice, descending axons project posteriorly toward the spinal cord at E12.5 (arrows). Both $Fzd3^{-/-}$ (***E***) and *Lp/Lp* (***F***, ***G***) mice display aberrant descending projections (arrows). $Fzd3^{-/-}$ mice form very short axons within the descending region, many of which project into inappropriate lateral and anterior directions (***E***). ***G***, *Lp/Lp* mice form axons within the descending region that display clear fasciculation defects and grow in large circles. Cell body orientation is also affected in $Fzd3^{-/-}$ mice. Although during their mediolateral migration most wild-type descending neurons display cell bodies oriented first laterally (en route to their final position) and then along the A–P axis as they reach their final lateral position (***H–J***), cell bodies in $Fzd3^{-/-}$ mice are oriented in several different directions, many projecting at an acute angle near the midline and failing to orient posteriorly as they reach their lateral resting position (***K***, ***L***). The cell orientation phenotype is schematized in ***N*** and ***O*** and quantified in ***P***. Graph shows average ± SEM. Almost 50% of $Fzd3^{+/+}$ mice are oriented at 180° compared with $Fzd3^{-/-}$ mice in which only ~10% of neurons are oriented posteriorly. M, Medial; L, lateral. Scale bars: ***A–C***, 50 μm; ***D–G***, 20 μm. $^{*}p < 0.05$, $^{**}p < 0.01$. Number of mutants analyzed: $Fzd3^{-/-}$; $n = 3$ for E11.5, $n = 15$ for E12.5; $n = 2$ for E13.5; $Celsr3^{-/-}$, $n = 3$ for E11.5; $n = 4$ for E12.5; *Lp/Lp*, $n = 4$ for E12.5.

Results

Serotonergic and dopaminergic neurons express core PCP components

In mouse, ascending 5-HT neurons are born between E11.5 and E12.5, just posterior to the isthmus, and are located close to the

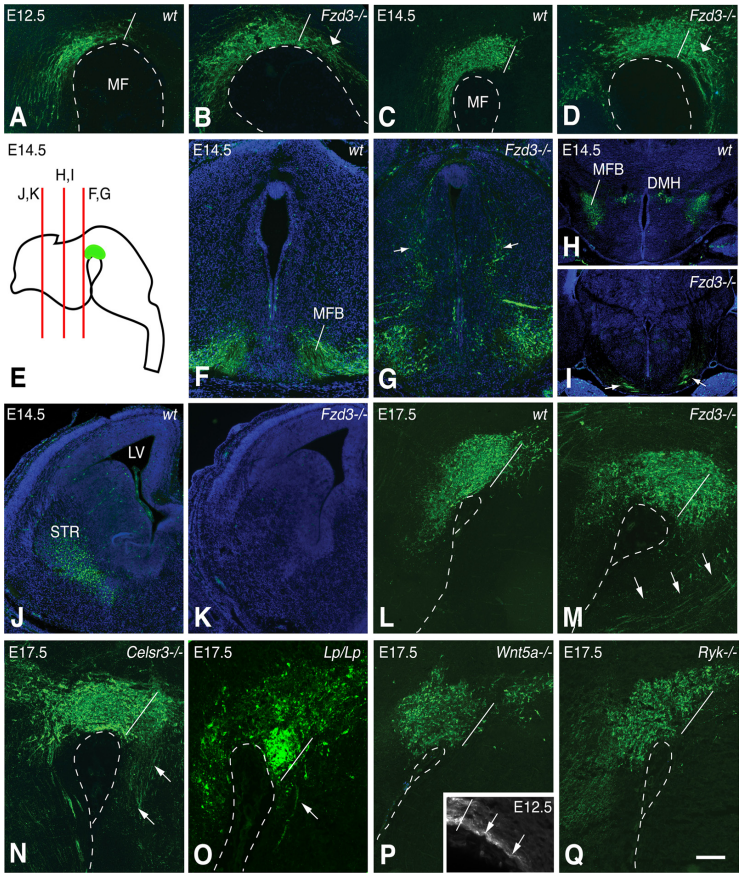

Figure 4. PCP mutant mice display axon guidance defects in the mdDA system. Immunohistochemistry for TH (in green) was used to analyze mdDA axon projections in *Fzd3*$^{-/-}$, *Celsr3*$^{-/-}$, *Lp/Lp*, *Wnt5a*$^{-/-}$, and *Ryk*$^{-/-}$ mice and littermate controls at E12.5 (***A***, ***B***), E14.5 (***C–K***), and E17.5 (***L–Q***). Coronal sections shown in ***F–K*** are counterstained with fluorescent Nissl to demarcate anatomical features. ***A–D*** and ***L–Q*** show sagittal sections. Whereas in wild-type (wt) control mice mdDA neurons project their axons anteriorly toward the forebrain, numerous TH-positive axons projecting posteriorly into the hindbrain region are found in *Fzd3*$^{-/-}$ mice at E12.5, E14.5, and E17.5 (***A–D***, ***L***, ***M***, arrows indicate posterior misprojections). Dotted line outlines mesencephalic flexure (MF) and white line the posterior boundary of the mdDA system. Schematic in ***E*** indicates the location of sections shown in ***F–K***. The medial forebrain bundle (MFB) is severely disrupted in *Fzd3*$^{-/-}$ mice, and ectopic axons can be found at dorsal levels in the brainstem but also near the midline (***F***, ***G***) and in abnormal ventral regions in the developing hypothalamus (DMH) near the optic chiasm (***H***, ***I***). In addition, TH-positive axons fail to reach their synaptic targets such as the striatum (***J***, ***K***) in *Fzd3*$^{-/-}$ mice. *Celsr3*$^{-/-}$ mice show a massive posterior backprojection of TH-positive axons at E17.5 (***N***). *Lp/lp* mice also show posterior projections, but this defect is less pronounced compared with *Fzd3*$^{-/-}$ and *Celsr3*$^{-/-}$ (***O***). In *Wnt5a*$^{-/-}$ mice, a small number of axons project posteriorly at E12.5 (inset) (supplemental Fig. S4, available at www.jneurosci.org as supplemental material), but at E17.5 this aberrant projection is no longer observed (***P***). *Ryk*$^{-/-}$ mice show normal TH-positive axon projections (***Q***). Number of mutants analyzed: *Fzd3*$^{-/-}$, $n = 13$ for E12.5; $n = 19$ for E14.5; $n = 8$ for E17.5; *Celsr3*$^{-/-}$, $n = 4$ for E17.5; *Lp/Lp*, $n = 2$ for E17.5; *Wnt5a*$^{-/-}$, $n = 4$ for E17.5; *Ryk*$^{-/-}$, $n = 3$ for E17.5. Scale bar: ***A–D***, ***F–K***, 100 μm; ***L–Q***, 50 μm.

midline (Fig. 1*A*,*B*, top red oval). They reside in six different nuclei (B4–B9) and send projections anteriorly to innervate various CNS structures. Shortly after the appearance of the ascending 5-HT neurons, descending 5-HT neurons migrate laterally in rhombomeres 5–7, become immunoreactive for 5-HT, and begin projecting axons posteriorly by E12.5 (Fig. 1*A*,*B*, bottom red oval). These neurons reside in three distinct nuclei (B1–B3) and project axons into the cervical spinal cord. Despite the well characterized anatomy of the 5-HT system, its axon guidance mechanisms, particularly along the A–P axis, are unknown. The first mdDA neurons, located just anterior to the isthmus, are born at approximately E10.5 and start to extend axons at E11.5. The mdDA system is composed of a medial population close to the midline, the VTA (A10), and a lateral subdivision, the SN (A9) (Fig. 1*A*,*B*, green oval), and both give rise to prominent ascending (anterior) axonal projections to the striatum and the medial prefrontal cortex.

It is evident that robust mechanisms must be in place to guide both hindbrain 5-HT and midbrain mdDA axons into their normal A–P directions. Wnts and their receptors have been implicated in determining the growth polarity of long descending and ascending spinal axon projections (Lyuksyutova et al., 2003; Liu et al., 2005; Wolf et al., 2008). In addition, Wnts and their receptors are expressed in the midbrain during early embryonic devel-

opment to regulate neuronal proliferation and differentiation (Andersson et al., 2008). Furthermore, in mice lacking Frizzled3, dopaminergic innervation of the striatum is lost (Wang et al., 2002). These results suggest that Fzd3 and associated signaling proteins may act to guide the growth of monoaminergic axons.

To test this hypothesis, we first examined the expression of core PCP components in the developing hindbrain by coimmunostaining against PCP proteins and 5-HT. We examined sagittal sections of the mouse hindbrain at E11.5 (supplemental Fig. S1, available at www.jneurosci.org as supplemental material) and E12.5, stages at which 5-HT neurons are born and begin pathfinding. We found that several PCP core components, Fzd3, Vangl2, and Celsr3, are expressed at E11.5 and E12.5 in both the ascending and descending 5-HT populations in cell bodies and fibers (Fig. 1*C*). Most, if not all, 5-HT-positive cells expressed Fzd3, Vangl2, and Celsr3. We also performed dissociated neuron cultures followed by immunocytochemistry and found that all 5-HT-positive cells express Fzd3 (Fig. 1*D*) and Celsr3 (Fig. 1*E*). To examine the expression of core PCP components in mdDA neurons, we used coimmunostaining against PCP proteins and TH on coronal sections. We focused on E11.5–E14.5, during which mdDA axon growth polarity is established. Fzd3, Celsr3, and Vangl2 are widely expressed in the midbrain region and in all TH-positive mdDA neurons as exemplified by E14.5 (Fig. 1*F*). Using dissociated neuron culture and immunocytochemistry, we confirmed that all TH-positive cells express Fzd3 and Ceslr3 (Fig. 1*G*,*H*). Unfortunately, the Vangl2 antibody did not stain well in the dissociated cultures. However, the immunostaining in Figure 1, *C* and *F*, clearly shows the presence of Vangl2 in 5-HT and TH neurons.

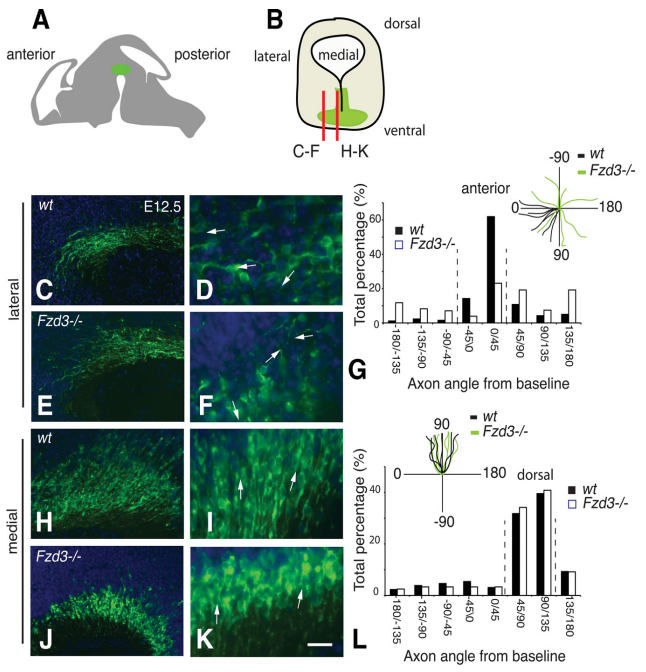

Figure 5. mdDA cell body orientation defects in *Fzd3* mutant mice. ***A***, ***B***, Sagittal and coronal view of the mdDA system (green). ***B***, Red lines indicate the location of ***C–F*** and ***H–K***. ***C–F***, ***H–K***, Immunohistochemistry for TH (green) of E12.5 sagittal sections. Sections are counterstained with DAPI in blue. In wild-type (wt) mice, most TH-positive neurons in the lateral mdDA system project their axons anteriorly (***C***, ***D***, arrows), whereas medially located neurons project dorsally (and then anteriorly) (***H***, ***I***). In contrast, in *Fzd3*$^{-/-}$ mutants, TH-positive neurons at lateral levels project their axons randomly, i.e., no longer confined to their normal anterior direction (***E***, ***F***). This effect is quantified as shown in ***G***. ***J***, ***K***, Neuronal orientation is essentially intact at medial levels in *Fzd3*$^{-/-}$ mutants. This is quantified as shown in ***L***. Quantifications shown in ***G*** and ***L*** represent angles between the lining of the mesencephalic flexure (baseline represents 0°) and the trajectory of the axon segment just proximal to the cell body. Insets show axon orientation plots for a few randomly selected axons. Scale bar: ***C***, ***E***, ***H***, ***J***, 25 μm; ***D***, ***F***, ***I***, ***K***, 12.5 μm.

PCP signaling is required for A–P guidance of serotonergic axons

To examine the potential role of PCP signaling in the development of the 5-HT system, we analyzed mouse lines deficient in PCP signaling components (Figs. 2, 3). Whole-mount immunostaining against 5-HT in the hindbrain reveals both the cell bodies and axons of serotonergic neurons (Figs. 2, 3). The *Fzd3*$^{-/-}$ mouse (Wang and Nathans, 2007) displays dramatic axon guidance defects in the 5-HT system at E12.5 (Fig. 2*A–C*). Although 5-HT-positive cell bodies appear to be in the appropriate A–P positions in *Fzd3*$^{-/-}$ mice, the axons in both the ascending and descending populations display marked misprojections. Axons in the ascending population project posteriorly and laterally instead of strictly anteriorly (Figs. 2*C*,*H*, 3*B*). At E12.5, when there are normally no axons present in R4, axons erroneously descend well into this rhombomere (Fig. 2*C*,*F*,*H*). The descending 5-HT population also displays striking axon guidance defects. Descending axons are distinctly shorter than their wild-type counterparts and project randomly along the A–P axis (Fig. 2*C*).

The *Looptail* mouse carries a mutation in the *Vangl2* protein that greatly affects Vangl2 stability and displays gross PCP defects (Montcouquiol et al., 2006). At E12.5, the *Lp* mouse displays axon guidance defects in both the ascending and descending populations (Figs. 2*D*,*I*, 3*C*). The ascending population displays a premature invasion of R4 and posterior projections (Fig. 2*D*,*F*,*I*). The descending population displays a striking over-fasciculation defect that is not present in the other PCP mutants we analyzed. Many of these axons grow together in tight bundles as if they were attracted to each other and appear much shorter (Fig. 2*D*). Higher-magnification images further reveal that these axons appear highly fasciculated (Fig. 3*F*,*G*). Like *Fzd3* mutants, very few axons project posteriorly into the cervical spinal cord in *Lp* mice.

We next examined 5-HT axon projections in *Celsr3*$^{-/-}$ mice (Tissir et al., 2005). Like the *Fzd3*$^{-/-}$ mouse, ascending axons in *Celsr3*$^{-/-}$ mice misproject both posteriorly and laterally (Fig. 2*E*, arrowhead). Axons also invade the R4 region at E12.5, although not as prominently as in *Fzd3* mutants (Fig. 2*E*,*J*). Although the descending population appears to show some orientation defects as observed in the *Fzd3*$^{-/-}$ mouse, many neurons form normal descending projections (Fig. 2*E*). This is in sharp contrast to the *Fzd3*$^{-/-}$ mouse, in which very few descending axons are formed at this stage.

At higher magnifications, we found that the cell bodies of wild-type neurons in the descending population are first oriented

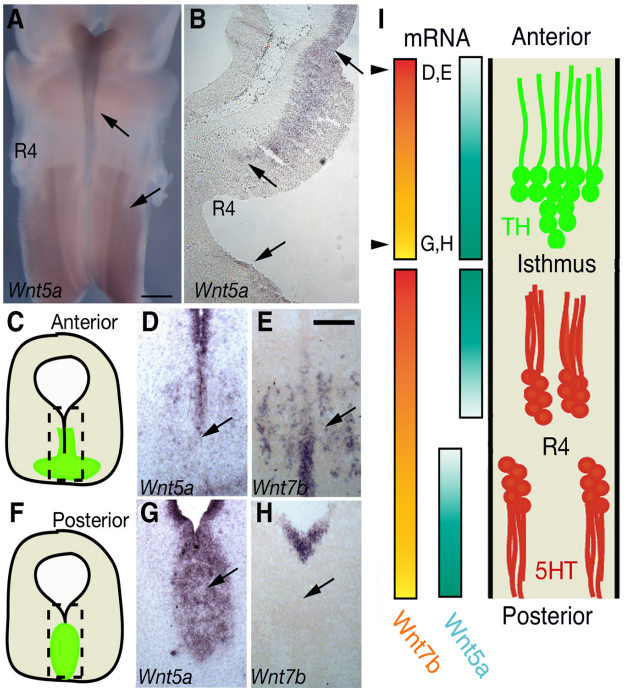

Figure 6. Multiple Wnts are expressed in gradients in both the hindbrain and midbrain. ***A***, Whole-mount *in situ* hybridization for *Wnt5a* in E12.5 mouse embryos. Arrows mark the locale of both ascending and descending 5-HT populations. ***B***, *In situ* hybridization for *Wnt5a* in a sagittal section of E12.5 hindbrain showing a anterior high, posterior low gradient near the midline and below the isthmus, consistent with the location of pathfinding ascending 5-HT axons at E12.5. ***C***, ***F***, Schematics indicating the location of images shown in ***D–H***. *In situ* hybridization for *Wnt5a* and *Wnt7b* in coronal (***D–H***) cryosections of the E14.5 midbrain region reveals a high posterior, low anterior expression gradient for *Wnt5a* and a high anterior, low posterior gradient for *Wnt7b*. ***I***, Proposed model for the role of Wnt/PCP signaling during A–P guidance of 5-HT and TH axons. 5-HT neurons (red) express the PCP components Fzd3, Celsr3, and Vangl2 to sense an attractive Wnt5a expression gradient in the hindbrain region directing ascending 5-HT axons anteriorly and descending 5-HT axons posteriorly. TH neurons (green) sense both an attractive Wnt 7b gradient and repulsive Wnt5a gradient guiding their axons anteriorly. Scale bars: ***A***, 500 μm; ***E***, 150 μm.

laterally while migrating, following the direction of their leading axons but eventually becoming oriented along the A–P axis as their axons turn posteriorly (Fig. 3*H–J*,*N*). However, in *Fzd3*$^{-/-}$ mice, many of the descending, but not ascending, cell bodies appear to remain oriented laterally or even slightly anteriorly. These cell bodies give rise to axons that grow out laterally before turning anterior or posterior (Fig. 3*K*,*L*,*O*). At E12.5, almost 50% of descending 5-HT neurons in *Fzd3*$^{+/+}$ mice are oriented at 180° compared with 10% in *Fzd3*$^{-/-}$ mice (Fig. 3*P*). Because during normal development axons are first oriented along the A–P axis before the soma rotates into its A–P position, it is plausible that the cell body follows the axon and uses it to position itself along the A–P axis (Hawthorne et al., 2010). Although our observations strongly support this model, we currently cannot exclude the possibility that PCP signaling may also regulate soma and dendrite orientation independent from A–P axon guidance.

Analysis of mice deficient for Ryk (Halford et al., 2000), another Wnt receptor, did not reveal obvious defects (supplemental Fig. S2, available at www.jneurosci.org as supplemental material). Similarly, genetic inactivation of Wnt5a (Yamaguchi et al., 1999), a known PCP Wnt, also did not appear to have a striking effect on 5-HT A–P axon guidance (supplemental Fig. S2, available at www.jneurosci.org as supplemental material).

PCP signaling is required for A–P guidance of dopaminergic axons

To assess whether Wnt/PCP signaling also controls mdDA axon patterning, we visualized mdDA axon trajectories in sections from *Fzd3*$^{-/-}$ mice using TH immunohistochemistry. At E11.5, many mdDA axons in *Fzd3*$^{-/-}$ mice follow an abnormal lateral trajectory at the level of the mdDA neuron pool (supplemental Fig. S3, available at www.jneurosci.org as supplemental material). At E12.5 and E14.5, a large number of mdDA axons are directed posteriorly in *Fzd3*$^{-/-}$ mice and aberrantly traverse the hindbrain (Fig. 4*A–D*). Although a subset of mdDA axons still projects anteriorly, presumably because of the presence of other A–P signals (Kolk et al., 2009; Yamauchi et al., 2009), this anterior projection is significantly reduced and disorganized in *Fzd3*$^{-/-}$ mice. In addition, many mdDA axons enter abnormal dorsal territories in the midbrain and diencephalon and aberrantly approach the midline (Fig. 4*E–G*). In the telencephalon, mdDA axons are displaced ventrally and are located in close proximity to the optic chiasm (Fig. 4*H*,*I*). No innervation of synaptic target regions such as the striatum or medial prefrontal cortex by mdDA axons is found at E14.5 or E17.5 (Fig. 4*J*,*K*) (data not shown).

Analysis of *Celsr3*$^{-/-}$ and *Lp* mice reveals similar abnormal posterior mdDA projections in the hindbrain at E12.5 and E17.5. At E12.5, *Fzd3*$^{-/-}$ and *Lp* mice show a similar, large number of posterior mdDA projections, whereas at this stage this defect is less pronounced in *Celsr3*$^{-/-}$ mice (supplemental Fig. S4, available at www.jneurosci.org as supplemental material). At E17.5, however, numerous mdDA axons are projecting into the hindbrain of *Fzd3*$^{-/-}$ and *Celsr3*$^{-/-}$ mice, but fewer posterior projections are present in *Lp* mice (Fig. 4*L–O*). In the diencephalon and telencephalon of *Celsr3*$^{-/-}$ mice, many mdDA axons follow aberrant lateral, dorsal, and ventral trajectories, as observed in *Fzd3*$^{-/-}$ mice. In contrast to *Fzd3*$^{-/-}$ mice, however, a small subset of mdDA axons reaches, but does not innervate, the striatum in E17.5 *Celsr3*$^{-/-}$ mice (supplemental Fig. S5*A*,*B*, available at www.jneurosci.org as supplemental material). mdDA axon projections in the forebrain of E17.5 *Lp* mice appear to innervate the striatum. However, it should be noted that *Lp* mice have an open neural tube and that the overall organization of the telencephalon is markedly changed in these mice (data not shown).

We also examined mdDA axon projections in *Wnt5a*$^{-/-}$ mice. In contrast to *Fzd3*$^{-/-}$, *Celsr3*$^{-/-}$, and *Lp* mice, *Wnt5a*$^{-/-}$ mice only display a minor and transient posterior projection of mdDA axons (Fig. 4*P*, inset). This suggests that the lack of Wnt5a signaling is compensated by other Wnt proteins at later developmental stages. We also analyzed mice deficient for the Wnt5a

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receptor Ryk. *Ryk*$^{-/-}$ mice did not show overt A–P guidance defects (Fig. 4*Q*). In addition, mdDA innervation of the striatum appears grossly normal at E17.5 in both *Wnt5a*$^{-/-}$ and *Ryk*$^{-/-}$ mice (supplemental Fig. S5*C–F*, available at www.jneurosci.org as supplemental material).

The descending 5-HT system shows defects in cell body orientation in *Fzd3*$^{-/-}$ embryos, which may be secondary to defective axon guidance (Fig. 3). Intriguingly, analysis of mdDA neurons at E11.5–E14.5 reveals similar defects. At E11.5, the overall cellular organization of the mdDA system is normal in *Fzd3* mutants (supplemental Fig. S3, available at www.jneurosci.org as supplemental material). At E12.5, mdDA cell bodies and axons in the most lateral aspect of the mdDA neuron pool of wild-type mice are oriented anteriorly (Fig. 5*A–D*,*G*). In contrast, mdDA cell bodies at the same anatomical location in *Fzd3*$^{-/-}$ mice are oriented in random directions following where the axons are oriented (Fig. 5*E–G*). mdDA neurons in the medial part of the mdDA system of wild-type mice send their axons dorsally before these axons are redirected anteriorly (Nakamura et al., 2000) (Fig. 5*H*,*I*,*L*). In contrast to lateral mdDA neurons, mdDA neurons in the medial part of the mdDA neuron pool in *Fzd3*$^{-/-}$ mice do not display obvious defects in cell body orientation (Fig. 5*J–L*). This difference between the lateral and medial mdDA neuron pool is reminiscent of what is observed for 5-HT neurons, with descending (lateral) but not ascending (medial) neurons showing cell body orientation defects (Fig. 3).

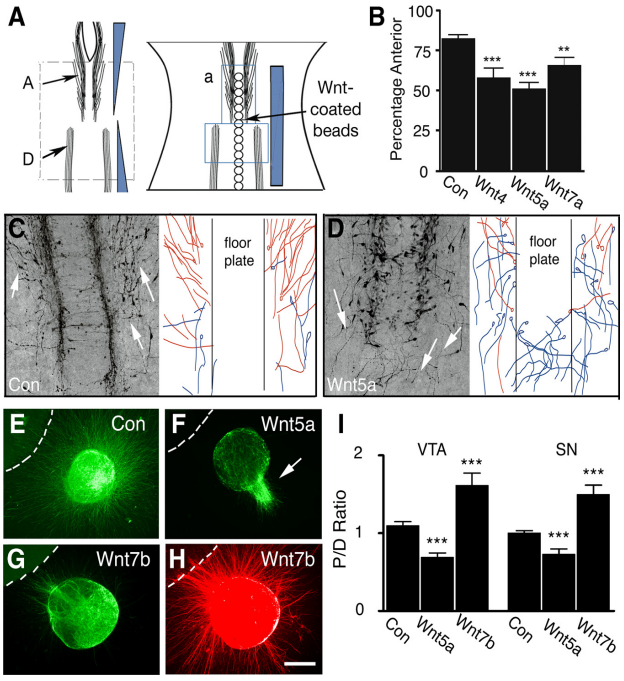

Figure 7. Both hindbrain serotonergic fibers and midbrain dopaminergic axons respond to Wnt proteins. ***A***, Wnt-coated beads were placed at the midline of E12.5 mouse hindbrain open-book preparations, cultured for 24 h, and subjected to 5-HT whole-mount staining. ***C***, ***D***, Addition of Wnt-coated beads to the midline disrupts the number of axons that project anteriorly in the ascending region. Graph in ***B*** shows average ± SEM of different experiments. Control, $n = 10$; Wnt4, $n = 6$; Wnt5a, $n = 10$; Wnt7a, $n = 9$. $^{*}p < 0.05$, $^{**}p < 0.001$, Student's *t* test. ***C***, Representative pictures of hindbrain explants showing the ascending 5-HT system as revealed by 5-HT immunostaining. Axons in control, H_2O-coated beads, project anteriorly (arrows, ***C***), whereas the presence of Wnt4-, Wnt5a-, and Wnt7a-coated beads at the midline alters the appropriate location and projections of these neurons. Drawings adjacent to the immunostainings in ***C*** and ***D*** are schematic representations of anteriorly (red) and posteriorly (blue) projecting 5-HT axons. ***E–H***, E12.5 VTA explants cultured adjacent to HEK293 cells (dotted line) secreting control protein (***E***; $n = 26$), Wnt5a (***F***; $n = 12$), or Wnt7b (***G***, ***H***; $n = 10$). Explants are coimmunostained for TH (green) and βIII-tubulin (red). ***I***, Quantification of the length of TH-positive neurites in the proximal (P) and distal (D) quadrants of culture assays as shown in ***E–H***. Graph shows average ± SEM P/D ratio. Note that, whereas Wnt5A repels TH-positive axons emanating from SN and VTA explants, Wnt7b attracts TH-positive axons (SN explants: control, $n = 9$; Wnt5a, $n = 9$; Wnt7b, $n = 9$). For each experimental condition, at least three independent experiments were performed. Scale bar: ***B***, ***D***, 100 μm; ***C***, ***E***, 50 μm; ***F–I***, 80 μm. $^{***}p < 0.001$, one-way ANOVA.

Wnts are expressed in anterior–posterior gradients in the basal midbrain and hindbrain

The only known ligands for PCP signaling are Wnt proteins. Using *in situ* hybridization, we sought to determine whether Wnts are expressed in the hindbrain and midbrain regions. Several Wnts were expressed in the developing hindbrain, but *Wnt5a* displayed the most striking expression pattern. In the hindbrain, we found that *Wnt5a* is expressed in an anterior high, posterior low expression pattern at the midline, where ascending serotonergic neurons are projecting anteriorly (Fig. 6*A*,*B*). Interestingly, the *Wnt5a* gradient direction switched from decreasing in the posterior direction to increasing at rhombomere 4 (Fig. 6*A*,*I*). It is therefore possible that Wnt5a attraction contributes to the proper A–P guidance of both ascending and descending 5-HT axons. In the midbrain of E12.5 and E14.5 embryos, we identified clear expression gradients for *Wnt5a* and *Wnt7b*. *Wnt5a* labels the medial part of the mdDA system (VTA) and displays a high posterior, low anterior expression gradient, with highest expression around the isthmus (Fig. 6*C*,*D*,*F*,*G*) (Andersson et al., 2008). This *Wnt5a* gradient flips at the isthmus and starts to decrease toward more posterior regions (Fig. 6*I*). *Wnt7b* is expressed in both the medial and lateral mdDA system and shows a high anterior, low posterior expression pattern in the midbrain (Fig. 6*E*,*H*). In all, these experiments show that Wnt proteins form expression gradients in the hindbrain and midbrain at the time 5-HT and mdDA axons are guided into the appropriate A–P direction.

Wnt proteins influence the orientation of 5-HT and mdDA axons and cell bodies

Because Wnts are expressed in gradients in the midbrain/hindbrain region, we tested whether Wnt proteins can direct the growth of 5-HT and mdDA axons. To determine whether Wnt ligands can direct 5-HT axons, we established a new hindbrain "open-book" explant culture. We added Wnt-coated beads along the midline to disrupt endogenous Wnt gradients (Fig. 7*A*). The exogenous presence of Wnt4, Wnt5a, and Wnt7a all affected the ability of axons in the ascending population to grow anteriorly

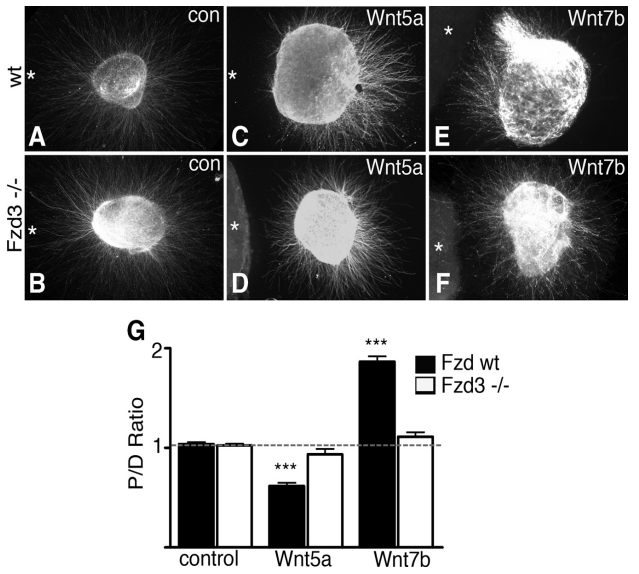

Figure 8. Dopaminergic neurons from *Fzd3* mutant mice are unresponsive to Wnt5a and Wnt7b. E12.5–E14.5 VTA explants from either wild-type (***A***, ***C***, ***E***) or *Frizzled3* mutant (***B***, ***D***, ***F***) mice were cultured adjacent to HEK293 cells (asterisks) secreting either control (con) protein [***A***, ***B***, $n = 19$ for wild-type (wt) and knock-outs; Wnt5a, ***C***, ***D***, $n = 16$ for wild-type and 18 for knock-outs; Wnt7b, ***E***, ***F***, $n = 17$ for wild-type and 18 for knock-outs]. Explants are immunostained for TH. ***G***, Quantification of the length of TH-positive neurites in the proximal and distal quadrants of culture assays. Graph shows average ± SEM P/D ratio. Dopaminergic axons from wild-type but not *Fzd3* mutant mice are repelled by Wnt5a and attracted by Wnt7b. For each experimental condition, at least three independent experiments were performed. Number of mice analyzed: $Fzd3^{-/-}$, $n = 6$; $Fzd3^{+/+}$, $n = 8$. Scale bar, 80 μm. ***$p < 0.001$ compared with control, one-way ANOVA. No statistically significant differences were found between knock-out Wnt5a and wild-type control or knock-out control or between knock-out Wnt7b and wild-type control or knock-out control.

toward the brain (Fig. 7*B–D*). Many of these axons now grew into the midline, posteriorly, or laterally, and fewer axons projected anteriorly (see blue misprojected axons in Fig. 7*D*). Some cultures also displayed 5-HT axons and cell bodies that were attracted to the midline after the addition of Wnt-coated beads (Fig. 7*D*). These defects correlate well with the misguidance phenotypes of ascending 5-HT neurons in PCP mutant mice. The placement of the Wnt-coated beads did not appear to have a robust effect on the descending population probably because of that fact that the descending axons and cell bodies are too far away from the midline, the site of bead implantation. These experiments suggest that Wnt5a serves as an attractive guidance cue for ascending 5-HT axons (Fig. 6*I*). To determine whether Wnts instruct mdDA axons to grow anteriorly, we used a previously established collagen matrix culture approach (Kolk et al., 2009). The chemotropic response of mdDA axons emanating from E12.5 SN or VTA explants to different Wnt proteins was tested. When confronted with cell aggregates releasing Wnt5a, TH-positive SN and VTA axons displayed strong chemorepulsive responses (Fig. 7*F*,*I*). In contrast, Wnt7b induced axon attraction of both mdDA axon populations (Fig. 7*G*,*I*). When examining all axons emanating from SN and VTA explants, as visualized by βIII-tubulin staining, no chemotropic effects were found, confirming the specificity of the response of TH-positive axons to Wnt5a and Wnt7b (Fig. 7*H*). These results together with the Wnt expression analysis suggest that Wnt5a functions to repel and Wnt7b to attract mdDA axons into their normal anterior direction in the midbrain (Fig. 6*I*).

Dopaminergic axons from *Fzd3* mutant mice are unresponsive to Wnts *in vitro*

Our results show expression of core PCP components in 5-HT and mdDA neurons and axon projections (Fig. 1) and reveal A–P guidance defects in 5-HT and mdDA axon pathways in PCP mutant mice *in vivo* (Figs. 2–4). The only known PCP ligands, Wnt proteins, are expressed in instructive gradients in the brainstem and provide directional cues for 5-HT and mdDA axons *in vitro* (Figs. 6, 7). Together, these data support the idea that PCP proteins serve as Wnt receptors on monoaminergic axons during A–P guidance. To further test this model, we performed collagen matrix assays with VTA explants dissected from *Fzd3* wild-type or mutant embryos. Fzd3 had been implicated as a neuronal receptor for Wnt3a and Wnt4 (Lyuksyutova et al., 2003; Endo et al., 2008). As described above, dopaminergic axons from wild-type explants were repelled by Wnt5a and attracted by Wnt7b (Fig. 8*A*,*C*,*E*,*G*). In contrast, axons emanating from dopaminergic explants obtained from $Fzd3^{-/-}$ mice did not display significant repulsive or attractive responses to Wnt5a and Wnt7b, respectively, compared with wild-type and knock-out control cultures (Fig. 8*B*,*D*,*F*,*G*). These results show that Fzd3 is an obligatory component of the receptor for Wnt5a and Wnt7b on dopaminergic axons and support the model that PCP receptors on monoaminergic axon projections function to sense instructive Wnt gradients in the brainstem, thereby controlling A–P axon guidance.

Discussion

Serotonergic and dopaminergic axon pathways mediate complex physiological functions and are therapeutic targets for many nervous system disorders. Their organization is exquisitely complex and requires a myriad of molecular signals for regulating different developmental steps. A crucial step in establishing monoaminergic connectivity is the initial orientation of 5-HT and mdDA axon projections along the A–P axis of the brainstem. Here we reveal a novel requirement for Wnt and PCP receptor proteins in the A–P guidance of 5-HT and mdDA axon projections. In PCP signaling mutants, both 5-HT and mdDA axons are severely misguided along the A–P axis (Fig. 9). Furthermore, we show that A–P cellular organization (i.e., cell body orientation) of monoaminergic nuclei is affected in Wnt/PCP mutants, without marked defects in the patterning of the brainstem (supplemental Fig. S6, available at www.jneurosci.org as supplemental material).

Wnt signaling controls A–P axon guidance beyond the spinal cord

Wnt family proteins have been identified as guidance cues that pattern axonal connections along the A–P axis in vertebrates

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and invertebrates (Zou, 2006; Zou and Lyuksyutova, 2007; Salinas and Zou, 2008). In vertebrate species, Wnts provide directional cues for ascending sensory and descending corticospinal axons along the A–P axis of the spinal cord (Lyuksyutova et al., 2003; Liu et al., 2005; Wolf et al., 2008). Until now, it was unknown whether A–P guidance in parts of the CNS outside the spinal cord also required Wnt signaling. In the present study, we show that indeed A–P guidance of 5-HT and mdDA axons depends on Wnt signaling. Both 5-HT and mdDA neurons and axons expressed PCP components (Fig. 1), and mice lacking these proteins displayed prominent A–P guidance defects in the midbrain and hindbrain (Figs. 2–4, 9). The only known PCP receptor ligands, Wnt proteins, were expressed in gradients in the brainstem and mediated guidance responses from 5-HT and mdDA axons in culture (Figs. 6, 7). mdDA neurons lacking the PCP receptor Fzd3 did not show significant axonal responses to Wnt5a and Wnt7b in culture (Fig. 8). These results establish that Wnt signaling through PCP pathway is a global A–P guidance mechanism that patterns axon tracts both in and outside the spinal cord. The marked defects in mdDA axon projections found in the telencephalon of *Fzd3* and *Celsr3* mutant mice (Fig. 4) support the idea that Wnt signaling may also control A–P guidance in the forebrain. In future studies, it will therefore be interesting to explore whether mdDA pathways or other longitudinal axon bundles in the forebrain are organized along the A–P axis by Wnt signaling.

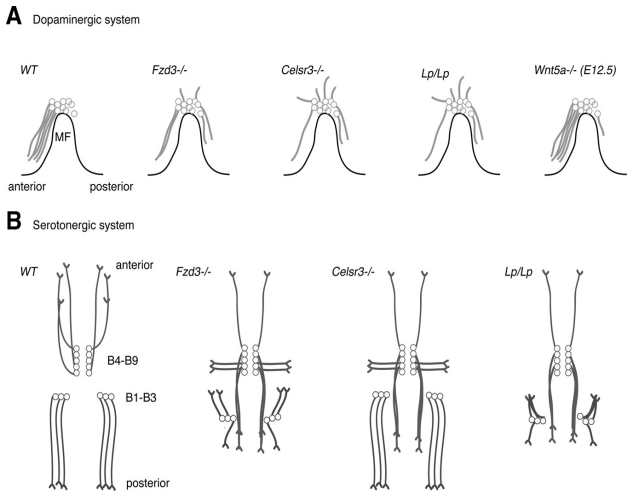

Figure 9. Axon guidance defects in the brainstem region of PCP and Wnt5a mutant mice. Schematic representation of the embryonic mesodiencephalic dopamine (mdDA) system (***A***, sagittal view) and ascending and descending serotonergic systems (***B***, horizontal view). ***A***, In wild-type (WT) mice, mdDA axon projections are oriented anteriorly. *Frizzled3*, *Celsr3*, and *Vangl2* (*Lp/Lp*) mutant mice display aberrant dorsal and caudal projections (from E12.5 until E17.5, the latest time point examined). *Wnt5a* mutant mice display a transient abnormal caudal projection at E12.5. ***B***, In wild-type mice, ascending serotonergic neurons (B9–B4) project their axons anteriorly, whereas descending neurons (B3–B1) give rise to posteriorly directed axon projections. *Fzd3* and *Vangl2* (*Lp/Lp*) mutant mice show ascending neurons that project their axons posteriorly or laterally and descending neurons that project axons anteriorly. *Celsr3* mutant mice predominantly show defects in ascending axon projections. Descending projections in *Vangl2* mice show hyperfasciculation phenotypes not found in *Fzd3* or *Celsr3* mutant mice.

Complexity of Wnt signaling during A–P guidance in the brainstem

Our study also reveals the remarkable complexity of Wnt signaling in the brainstem. Wnt gradients in the brainstem are not unidirectional, and the same Wnt gene (e.g., *Wnt5a*) can display opposite gradients anterior or posterior to morphologically recognizable boundary structures, such as the isthmus, or morphologically nonrecognizable but molecular defined structures, such as rhombomere 4 (Fig. 6). Furthermore, the same mdDA neurons can respond differently to different Wnts, which are expressed in opposite gradients in the midbrain (attraction by Wnt7b and repulsion by Wnt5a) to ensure correct A–P guidance (Fig. 7). In addition, neighboring neuronal populations (mdDA and 5-HT) respond differentially to the same Wnt protein (Wnt5a repels mdDA axons but attracts 5-HT axons) (Fig. 7). The molecular mechanism that underlies this difference in Wnt5a responsiveness is currently unknown but is likely to involve additional receptor proteins.

There may also be redundancy in the PCP signaling components involved in 5-HT and mdDA A–P guidance. *Fzd3* mutants show broad and strong defects. In contrast, mutations in *Celsr3* and *Vangl2* result in less severe or partial phenotypes. For example, descending 5-HT neurons show much weaker phenotypes in axon guidance and cell body defects in *Celsr3*$^{-/-}$ compared with *Fzd3*$^{-/-}$ mice (Fig. 2). Potentially, other members of the *Celsr* family, *Celsr1* or *Celsr2* (Tissir et al., 2002), compensate for the loss of function of *Celsr3*. The persistence of a small number of appropriate 5-HT and mdDA A–P projections in different PCP mutants further indicates that, in addition to PCP signals, other molecular cues are involved in A–P guidance in the brainstem (Kolk et al., 2009; Yamauchi et al., 2009). Finally, *Looptail* mice display a severe hyperfasciculation phenotype that is not found in *Fzd3*$^{-/-}$ and *Celsr3*$^{-/-}$ mice, suggesting that Vangl2 may also control axon–axon interactions (Fig. 3).

Although there is a transient defect in mdDA axon projections in *Wnt5a*$^{-/-}$ embryos, we did not observe a similar phenotype in 5-HT axon projections. In addition to *Wnt5a*, *Wnt4* and *Wnt7b* are expressed in the floor plate. The Wnt7b and Wnt5a gradients follow the same direction at the level of the ascending 5-HT system, and both Wnts appear to attract ascending 5-HT axons. Thus, Wnt7b may compensate for the loss of Wnt5a (Fig. 6). It is interesting that, although Wnt7b may attract mdDA axons anteriorly, we still observed a transient TH axon phenotype in the absence of Wnt5a (Fig. 4). It is possible that mdDA axons depend more on Wnt5a repulsion than Wnt7b attraction for anterior growth or perhaps the Wnt5a gradient is established first to repel mdDA axons anteriorly, after which the Wnt7b gradient is formed slightly later to further attract mdDA axons anteriorly (Fig. 6). In this model, mdDA axon projections may eventually become corrected by the Wnt7b gradient in *Wnt5a*$^{-/-}$ mice. Alternatively, aberrant axons in *Wnt5a*$^{-/-}$ mice may not survive and die back at later developmental stages. Future studies will address these and other possible mechanisms.

Wnt/PCP signaling dictates cell body orientation along the A–P axis

Neural circuit construction involves the proper orientation of axons, dendrites, and cell bodies. Because of their well documented anatomical organization, dopaminergic and serotonergic nuclei comprise an advantageous system for studying molecular cues that establish the polarity of neurons and their projections during development (Rubenstein, 1998; Goridis and Rohrer, 2002; Cordes, 2005; Smidt and Burbach, 2007; Van den Heuvel and Pasterkamp, 2008). In this study, we report that Wnt signaling is required for two different aspects of monoaminergic circuit organization. First, Wnt signaling controls the A–P guidance of monoaminergic axons. Second, proper Wnt signaling is also required for the correct cell body orientation of both 5-HT and mdDA neurons in the brainstem, which is disrupted in *Fzd3* mutant mice. We found that, at least for 5-HT neurons, axon guidance appears to precede the final orientation of the soma. In wild-type animals, cell bodies of migrating, descending 5-HT neurons initially point laterally but begin to reorient along the A–P axis as they approach their final lateral position, thereby obeying the direction of their axon projections (Fig. 3*N*). Recent work by Hawthorne et al. (2010) uncovers similar mechanisms and indicates that early-differentiated 5-HT neurons migrate ventrally from the ventricular zone to the pial surface by somal translocation. During somal translocation, neurons maintain a bipolar morphology, and the soma translocates along primitive ventricular and pial processes. The lateral migration of descending 5-HT neurons observed in our open-book preparations is reminiscent of somal translocation, with the axon being the leading process through which the cell body translocates. This would suggest that the direction of the axon projections dictate cell body orientation. However, one cannot exclude the possibility that cell body orientation and thus dendrite orientation may also be regulated independently from the axons by Wnt/PCP signaling. Future studies combining time-lapse imaging (Hawthorne et al., 2010), fluorescently labeled mouse lines [e.g., ePet–EYFP lines (Scott et al., 2005)], and our newly developed open-book cultures will be required to further unveil the specific mechanisms underlying cell body reorientation.

mdDA neurons are born in the ventricular zone of the mesodiencephalon and migrate ventrally along the processes of radial glia to ultimately form the SN and VTA (Smidt and Burbach, 2007; Tang et al., 2009). In PCP mutants, ventral (radial) migration of mdDA neurons is intact, but, in the most distal part of this migratory process, the orientation of mdDA cell bodies that will eventually occupy more lateral domains (e.g., the SN) is perturbed (data not shown). One model to explain these defects, based on our observations in the descending 5-HT system, is that the final stage of mdDA neuron migration, but not the radial migration, is guided by PCP signaling. Similar to what we suggest for the more laterally located descending 5-HT neurons, PCP signaling may indirectly influence mdDA neuron cell body orientation by controlling axon guidance. Future experiments are needed to address these and other possibilities.

Concluding remarks

Here we show that Wnt/PCP signaling determines the proper A–P organization of ascending and descending serotonergic and dopaminergic pathways in the brainstem. Although Wnts had been reported previously to regulate the generation and differentiation of mdDA neurons (Andersson et al., 2008), our work establishes that these proteins are reused as guidance cues for monoaminergic axons at subsequent developmental stages. This finding unveils a previously poorly characterized aspect of mdDA pathway formation and identifies one of the first guidance mechanisms involved in 5-HT circuitry formation *in vivo*. Furthermore, this is, to our knowledge, the first example of a role for Wnt/PCP signaling in A–P axon guidance outside the spinal cord.

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Chapter 4

Frizzled3 regulates the topology of the developing mesodiencephalic dopamine (mdDA) system

Asheeta A. Prasad, Youri Adolfs and R. Jeroen Pasterkamp

Abstract

Wnt/Frizzled signaling controls an array of cellular processes in the developing and mature brain. Within the mesodiencephalic dopamine (mdDA) system, Wnts and their receptors have been shown to contribute to the regulation of several developmental processes including neuronal differentiation and axon growth and guidance. However, the receptors that mediate the effects of Wnts during early stages of mdDA system development have remained largely unknown. Here we show that *Fzd3* is expressed in and around mdDA neurons during the time these neurons migrate and differentiate. In *Fzd3* -/- mice, the topological organization of mdDA neurons within the mesodiencephalon along the rostrocaudal and medio-lateral axes was significantly altered. Similar defects were observed in *Wnt5a* and *Celsr3*-/- mice. Furthermore, in *Fzd3*-/- the radial glia scaffold used for migration of mdDA neurons had significantly widened. Overall, these results suggest that Fzd3, Celsr3 and Wnt5a cooperate in the patterning of the mdDA neuron pool and that defects in the organization of the radial glia scaffold may underlie the abnormal lateral positioning of mdDA neurons.

Introduction

Wnts form a large family of secreted cysteine-rich glycoproteins with 19 members in vertebrate species (Miller 2002). Wnts are extremely pleiotropic and important for the regulation of a wide range of cellular processes in the brain (Ciani and Salinas et al, 2005). These include various developmental processes such as neuronal specification/differentiation (McMahon and Bradley, 1990), axon outgrowth (Li et al, 2009), and axon guidance (Keeble et al, 2006, Zaghetto et al, 2007). The cellular effects of Wnt proteins can be mediated by several different cell surface receptors and downstream signaling mechanisms. Receptors for Wnts in neurons include members of the Frizzled, Lrp, Ryk and Ror receptor families (Kikuchi et al, 2006; Ciani and Salinas, 2005). Downstream Wnt signaling pathways can be divided into canonical and non-canonical pathways. The non-canonical pathway is further subdivided into the Wnt/Calcium and Wnt/planar cell polarity (PCP) pathway (Ciani and Salinas, 2005).

Dopaminergic neurons that originate from the mesodiencephalon (mdDA neurons) form complex neuronal connections within the forebrain to control motor and cognitive functions (Prasad and Pasterkamp, 2007; Van den Heuvel and Pasterkamp, 2008). During development of the mdDA system, Wnts have been shown to contribute to the patterning, specification, proliferation, neurogenesis, and survival of mdDA neurons (Castelo-Branco & Arenas, 2006). Several *Wnt5a*-/- mice have been generated and analyzed for defects in the mdDA system. Thus far, the phenotypes observed in the mdDA system of *Wnt1* and *Wnt5a*-/- mice are most severe. Deletion of *Wnt1* causes a loss of mdDA neurons (Danielian and McMahon, 1996) and *Wnt5a*-/- mice show disturbed midbrain morphogenesis (Andersson et al, 2008). However, the Wnt receptors and signaling pathways that mediate the effects of Wnt1 and Wnt5a during mdDA system development remain to be identified. Frizzled3 (Fzd3) is prominently expressed in mdDA neurons and *Fzd3*-/- mice display defects in mdDA axon guidance (Wang et al, 2002, Fenstermaker et al, 2010). During mdDA axon guidance, Fzd3 functions as an axonal receptor for Wnt5a supporting the hypothesis that Fzd3 may also mediate the effects of this Wnt during earlier stages of mdDA neuron development (Fenstermaker et al, 2010). In mdDA axon guidance, Fzd3 utilizes PCP signaling. Fzd3/PCP signaling is involved in facial branchiomotor neuron migration and the planar orientation of inner ear sensory hair (Wang et al, 2006; Vivancos et al, 2009). Fzd3 has recently also been implicated in the neurogenesis and target innervation of sympathetic neurons independent of PCP signaling (Armstrong et al, 2011). PCP is the process involved in assembling the orientation of asymmetric structures within a plane (Wang et al, 2006). PCP signaling also regulates convergent extension (CE), which is the coordinated movement of cells within a 3-dimensional structure. This process controls the formation of converged structures such as the anterioposterior axis. Cells converge along one axis (anterioposterior) and elongate along the perpendicular axis via cellular movement (Montcouquiol et al, 2006). Whether these or other functions of Fzd3 contribute to the morphogenesis of the mdDA system is unknown.

In the present study, we examined the role of Fzd3 in the development of the mdDA system. We report that in *Fzd3*-/- mice the organization of mdDA neurons within the mesodiencephalon is perturbed. The mdDA system in *Fzd3*-/- mice exhibited a lateral

and caudal expansion of mdDA neurons. In contrast, the overall organization of the mesodiencephalon was intact as revealed through the analysis of several molecular markers. Mouse mutants for other PCP proteins such as Celsr3 and Wnt5a displayed similar phenotypes. Interestingly, in *Fzd3*-/- mice the radial glia scaffold used for the migration of mdDA neurons was significantly widened. We propose that this broadening of the radial glia scaffold causes the lateral mispositioning of mdDA neurons. Together, these data identify a novel role for Fzd3 and related PCP proteins in the cellular organization of the mdDA system.

Materials and Methods

Animals

All animal use and care were in accordance with institutional guidelines. C57BL/6 mice were obtained from Charles River. *Fzd3* mutant mice were obtained from Jeremy Nathans (Johns Hopkins University School of Medicine, Baltimore, USA (Wang et al., 2002)). *Celsr3* mutant mice were received from Fadel Tissir and Andre Goffinet (Université Catholique de Louvain, Institute of Neuroscience, Brussels, Belgium (Tissir et al, 2005)). *Ryk* mutant mice were from Steven Stacker (Ludwig Institute for Cancer Research, Melbourne, VIC, Australia (Halford et al., 2000)). *Wnt5a* mutant mice were purchased from the Jackson Laboratory. Timed-pregnant mice were killed by means of cervical dislocation. The morning on which a vaginal plug was detected was considered embryonic day 0.5 (E0.5).

In situ hybridization

For *in situ* hybridization and immunohistochemistry, E11.5-E17.5 brains were fixed in 4% paraformaldehyde (PFA) for 30 minutes to 1 hour at room temperature (RT), followed by immersion in 30 % sucrose. Brains were frozen on powdered dry ice, cryosectioned, mounted on Superfrost Plus slides (Thermo Fisher Scientific), air-dried, and stored desiccated at -80^{0}C. Non-radioactive *in situ* hybridization on cryosections (14 µm) was performed as described in Chapter 4 of this thesis. In brief mouse *Fzd3* cDNA for digoxigenin-labeled probes was generated by RT-PCR from adult mouse whole brain RNA using the following primers; *Fzd3* forward primer: GCGGTTGATGGAGTTGCTAT, and *Fzd3* reverse primer: TGGCAGAGAGGCAAATGAT. *Fibroblast Growth Factor 8 (Fgf8)* cDNA was generated with the following primers; *Fgf8* forward primer: GCAAGGACTGCGTATTCACA and *Fgf8* reverse primer :GGTAGTTGAGGAACTCGAAGC. *Tyrosine hydroxylase*, *Forkhead box A2 (FoxA2)* and *Aldehyde dehydrogenase family 1, subfamily a1 (Adh2)* cDNAs were as described previously (Smits et al., 2005). DIG-labeled RNA probes were generated as described before (Pasterkamp et al., 2007). Sense controls were performed in parallel to assess non-specific labeling. Staining was visualized using a Zeiss Axioskop 2 microscope.

Immunohistochemistry

Brain tissue was processed as described for *in situ* hybridization. Sections were washed in phosphate-buffered saline (PBS), pH 7.0. Non-specific antigens were blocked with block-

ing solution [3% Normal Goat Serum, 1% Normal Horse Serum, 1% Normal Donkey Serum, 1% Bovine Serum Albumin, 1% Glycine, 0.1% Lysine, 0.4% Triton-X-100 in PBS] for 30 minutes at RT. Primary antibodies were incubated overnight in blocking buffer at 4^0C. The primary antibodies used were: rabbit anti-TH (1:1000, Pel-Freeze) and mouse-anti-nestin (1:100, DSHB). The following day, sections were washed in PBS and incubated for 1 hour at RT with the appropriate AlexaFluor-labeled secondary antibodies. Sections were washed in PBS, counterstained with fluorescent DAPI (Sigma), washed extensively and embedded in Mowiol (Sigma). Staining was visualized using a Zeiss Axioskop 2 microscope.

Quantification of lateral expansion
Coronal sections from E14.5 *Fzd3* wt and *Fzd3*-/- mice were immunostained with anti-TH antibody and captured and analyzed using Zeiss Axioskop 2 software. The angle measuring tool was used to quantify the lateral expansion observed in *Fzd3*-/- mice. Two lines were drawn to determine angles; one vertical line started at the midline and running down into the mdDA system. A second line also started at the midline but connected to the most lateral aspect of the TH-positive region (n=3 embryos per genotype).

Analysis and quantification of changes in the radial glia scaffold
Coronal sections co-immunostained with anti-nestin and anti-TH antibodies were analyzed using Zeiss Axioskop 2 software. First, images were captured using a 10x magnification using a Zeiss Axioskop 2 microscope. Using the angle-measuring tool, the starting plane was marked by a vertical line starting at the midline and running down into the mdDA system. A second line was drawn at the initiation of the radial glia scaffold (at the ventricular zone) and followed individual radial glia fibers into the mdDA system (n=30 radial glia in three different wt and *Fzd3*-/- embryos each).

Results

Fzd3 is expressed in the mdDA system during early development

The first mdDA neurons are born around E11 in the ventricular zone of the mesodiencephalon followed by a ventral migration of these cells in the subsequent days (Burbach and Smidt, 2007). By E11.5 the first mdDA neurons start to extend neurites and by E14.5 mdDA axons innervate target regions such as the striatum (Kolk et al, 2009; Van den Heuvel and Pasterkamp, 2008). To study the role of Fzd3 in the development of mdDA neurons, we examined the expression of Fzd3 from E11.5 to E14.5 in the mdDA system (Fig. 1). We conducted *in situ* hybridization for *Fzd3* in combination with immunohistochemistry for tyrosine hydroxylase (TH), as a marker for mdDA neurons, on coronal sections. At E11.5, *Fzd3* was expressed abundantly in cells in the mesodiencephalon including in the small number of TH+ neurons present at this stage (Fig.1 A-C). Interestingly, at E12.5 and E14.5, *Fzd3* was expressed in a low rostral to high caudal pattern in the region occupied by mdDA neurons (Fig.1 D-O). *Fzd3* co-localized with TH in the medial part of the caudal mesodiencephalon but not at more rostral levels. In addition, *Fzd3* was expressed in non-mdDA (TH-) neurons. In all, *Fzd3* is expressed in and around

the developing mdDA neuron pool at the time this system is formed. Fzd3 mutant mice display a lateral expansion of both mdDA cell bodies and axons Since *Fzd3* was expressed during early stages of mdDA system development, we next examined the functional role of Fzd3 during mdDA system development by analyzing *Fzd3*-/- mice and littermate controls using TH immunohistochemistry. In E11.5 wild-type (wt) embryos all mdDA axons were orientated in the rostral direction and therefore not visible in the plane of a coronal section (Fig.1A). In contrast in *Fzd3*-/- mice a few TH+ axons were projecting laterally (Fig.1B). By E14.5, both TH+ axons and cell bodies showed a prominent lateral expansion in *Fzd3*-/- mice at all rostrocaudal levels examined (Fig. 1C-H).

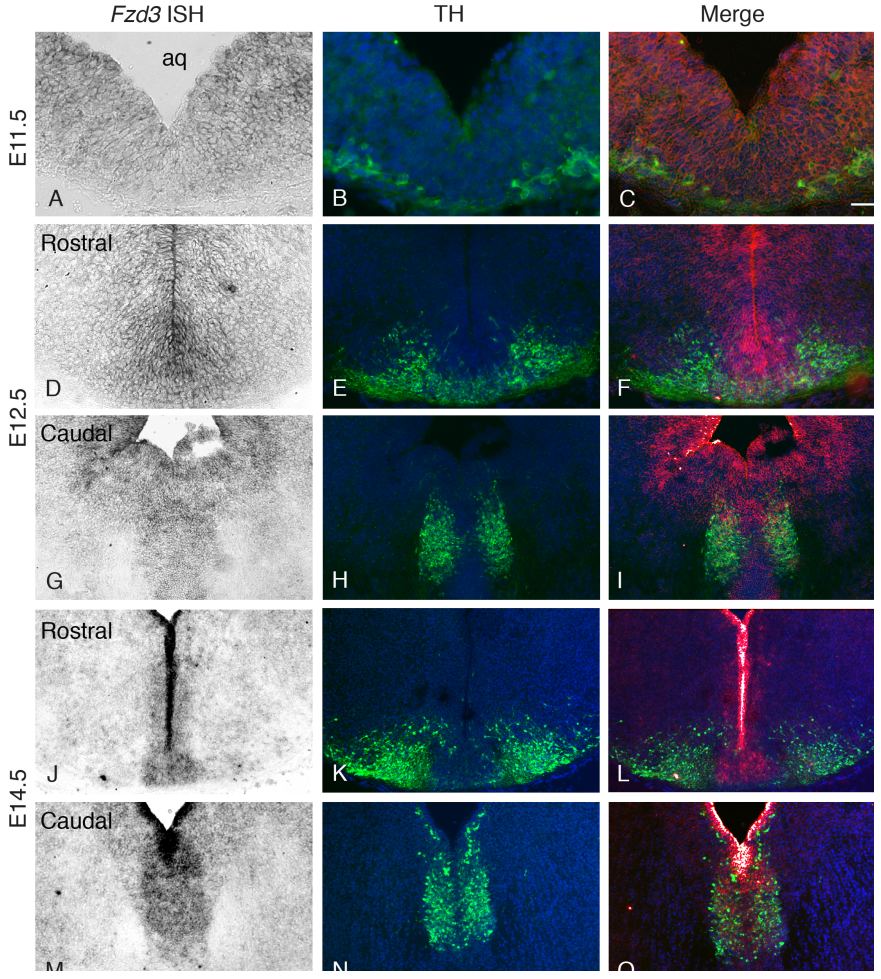

Figure 1. Fzd3 shows a highly specific pattern of expression in the embryonic mesodiencephalic dopamine (mdDA) system.

In situ hybridization for *Fzd3* in combination with immunohistochemistry for tyrosine hydroxylase (TH) in green on cryosections at the level of the E11.5 (A-C), E12.5 (D-I), and E14.5 (J-O) mdDA system. The *in situ* signal has been pseudo-colored to show overlap of *Fzd3* and TH expression. At E12.5 and E14.5, sections of the rostral and caudal aspect of the mdDA system are shown. Note that *Fzd3* co-localizes with TH in the medial aspect of mdDA system at caudal but not rostral levels. Fzd3 is expressed in TH+ mdDA neurons as well as in non-mdDA (TH-) neurons. *aq*: aquaduct. Scale bar=80 mM (A-C), 40 mM (D-O)

The organization of the medial aspect of the mdDA system appeared unchanged. To quantify this defect, the span of the mediolateral area of TH expression was measured in coronal sections from *Fzd3*+/+ and *Fzd3*-/- brains at E14.5 (Fig. 2I,J). In line with our qualitative observations, an increase of 28% was observed in the lateral extent of the mdDA system in *Fzd3*-/- as compared to *Fzd3* wt mice (Fig. 2I,J).

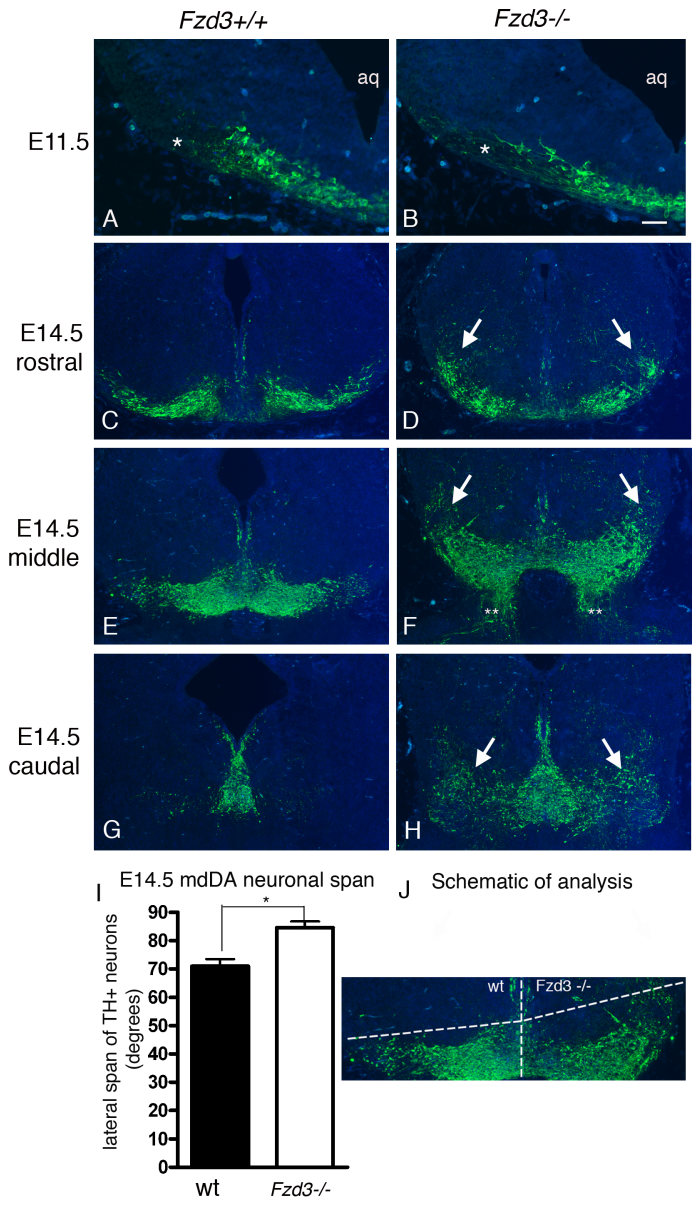

Figure 2. Fzd3 deficient mice display a lateral expansion of the meso-diencephalic dopamine (mdDA) system. Immunohistochemistry for tyrosine hydroxylase (TH, in green) was used to analyze the distribution of mdDA neurons and axons in *Fzd3* -/- mice and littermate controls at E11.5 (A, B) and E14.5 (C-H). (A, B) At E11.5, the cellular organization of the mdDA system is intact in *Fzd3*-/- mice. However, a small number of TH+ axons projects laterally in the absence of Fzd3 (asterisks). From E12.5 onwards, TH signals could be observed lateral to the normal location of mdDA neurons and axons in *Fzd3*-/- mice (C-H, white arrows). (I) This defect is especially pronounced at caudal levels and represents a lateral expansion of 28% as compared to wt littermates. Double asterisks in F indicate abnormal caudal projections (see Chapter 3). (J) Method used to quantify the lateralization defects. aq, aquaduct. *p < 0.05, one-way ANOVA. Scale bar=80 mM (A-B), 40 mM (C-H)

TH immunohistochemistry labels both mdDA cell bodies and axons. To clarify whether mdDA axons and/or neuronal bodies were disorganized in *Fzd3*-/- mice, we performed *in situ* hybridization for *TH* to label cell bodies only. Analysis of coronal sections at the

rostral, middle and caudal regions of the mdDA system revealed a similar lateralization phenotype as observed when using TH immunohistochemistry. At each level, compared to *Fzd3*+/+, *Fzd3*-/- embryos showed a lateral expansion of TH+ neurons.
At E14.5, the nicely compacted mdDA neuron pool observed in *Fzd3*+/+, (Fig. 3A-C) was dispersed in *Fzd3*-/- embryos (Fig. 3D-F). Analysis of sagittal sections subjected to *in situ* hybridization for *TH* revealed another defect in *Fzd3*-/- embryos. At caudal levels a prominent increase in the number of TH+ neurons was observed in *Fzd3*-/- but not *Fzd3*+/+, mice, whereas neuronal numbers at rostral levels appeared normal. In addition, the mdDA system occupied a larger rostrocaudal region along the mesencephalic flexure (Fig. 3G, H). In all, *Fzd3* deficiency leads to an increase in the number of TH+ neurons and axons in the mediolateral and rostrocaudal domains, indicating a role in the organization of the mdDA system.

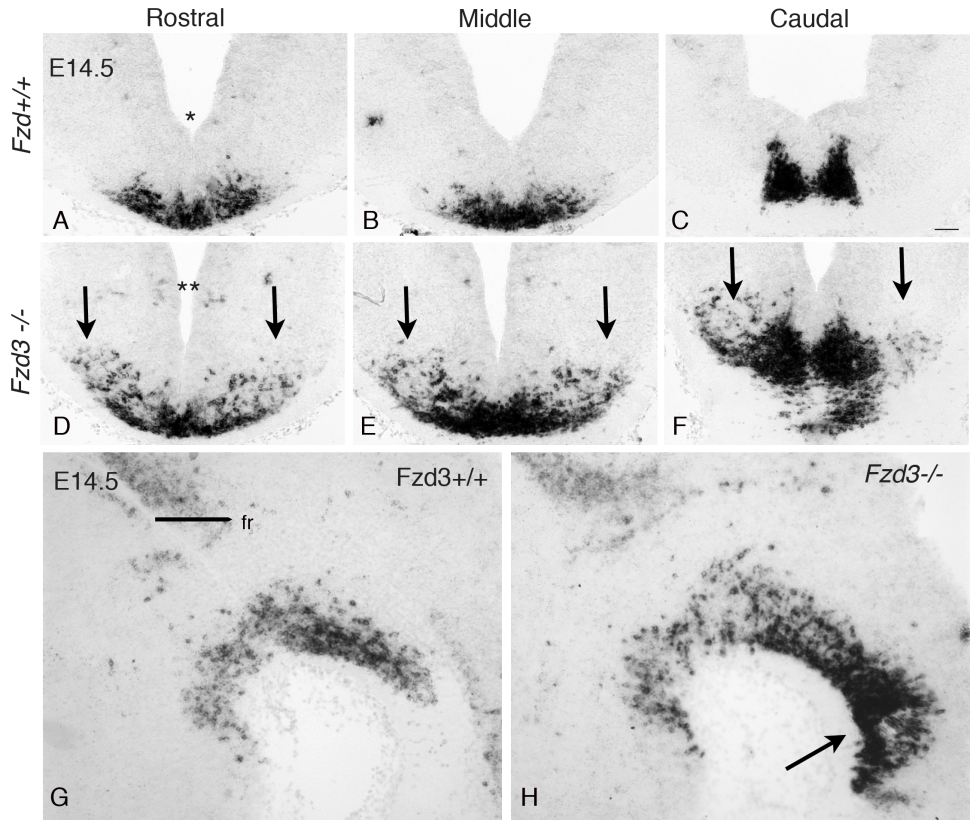

Figure 3. Fzd3 deficient mice display a lateral and caudal expansion of mesodiencephalic dopamine (mdDA) neurons.
In situ hybridization for *tyrosine hydroxylase* (*TH*) on sections from E14.5 *Fzd3*-/- mice and littermate controls to localize TH+ cell bodies. A-F shows coronal sections at rostral, middle and caudal levels in the mdDA system. G and H show sagittal sections at the level of the fasciculus retroflexus (fr). (A-F). A lateral expansion of TH+ neurons is found at all rostrocaudal levels in *Fzd3*-/- mice (black arrows). (G, H) A significant increase in the number of *TH*+ neurons is observed in the caudal mdDA system in *Fzd3*-/- mice (black arrow in H). Note, that *Fzd3*-/- mice show a narrowing of the aquaduct (see asterisk in *Fzd3*+/+ (A) and double asterisks in *Fzd3*-/- (D). Scale bar=40 mM (A-F), 80 mM (G,H).

The overall organization of the mesodiencephalon is intact in *Fzd3*-/- mice.
The defects observed in the organization of the mdDA system in *Fzd3*-/- mice can be the result of an abnormal positioning of mdDA neurons and/or of more general changes in the midbrain region. For the proper organization of the midbrain, dorsoventral (D-V) and anteroposterior (A-P) patterning is essential. Therefore we used several different molecular markers to assess the overall organization of the midbrain region. To examine D-V patterning in *Fzd3*-/- mice we combined immunohistochemistry for TH with *in situ* hybridization for *Forkhead box A2 (FoxA2)*. *FoxA2* expression is limited to the floor and basal plates in the developing mdDA region. No significant change in *FoxA2* expression was observed in *Fzd3*-/- mice as compared to control (Fig. 4A-D).

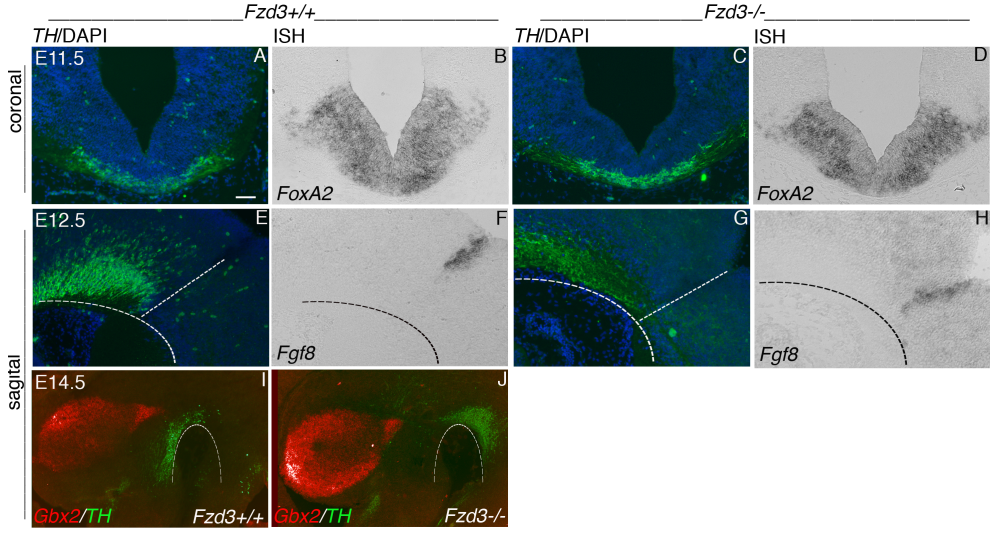

Figure 4. The overall anatomical organization of the midbrain region is intact in Fzd3 mutant mice.
Immunohistochemistry for tyrosine hydroxylase (TH,in green) in combination with *in situ* hybridization for molecular markers. (A-D) Expression of *FoxA2* in the floor and basal plate is unchanged in *Fzd3*-/- mice. (E-H) *Fgf8* expression in the isthmus marks the caudal boundary of the mdDA system. In *Fzd3*-/- mice, TH+ neurons have expanded caudally but not beyond the isthmus. (I, J) At E14.5, *Gbx2* mRNA is expressed in prosomere 2, rostral to the mdDA system. In E14.5 *Fzd3*-/- mice, the expression of TH has shifted caudally, but expression of *Gbx2* is unchanged. Scale bar=80 mM (A-H), 40 mM (I, J).

The isthmus is a molecular boundary specifying the mdDA neuron field (Smidt and Burbach, 2007). It is located caudally to the mdDA neuron pool and secretes Fibroblast Growth Factor 8 (Fgf8) (Crossley and Martin, 1995). Since a caudal shift in the distribution of mdDA neurons was observed in *Fzd3*-/- mice, we hypothesized that TH+ neurons may no be longer restricted to the region caudal to the isthmus. To test this hypothesis, *in situ* hybridization for *Fgf8* was combined with TH immunohistochemistry on sagittal sec tions from *Fzd3* wt and *Fzd3*-/- embryos. In wt embryos, TH+ neurons were always distributed caudal to the region of *Fgf8* expression (the isthmus). A similar distribution of TH+ neurons was observed in *Fzd3*-/- mice (Fig. 4E-H).
Gastrulation brain homeobox 2 (Gbx2) is a homeodomain transcription factor that labels prosomere 2. Prosomere 2 is an anatomical division between the pretectum and

prethalamus, serving here as a rostral marker for the mdDA system. We combined *in situ* hybridization for *Gbx2* with immunohistochemistry for TH. *Gbx2* expression was similar in E14.5 *Fzd3*+/+ and *Fzd3*-/- embryos (Fig. 4I, J). Overall, these results suggest that the overall organization of the midbrain region in relation to the mdDA system is undisturbed in *Fzd3*-/- mice.

The lateral mdDA neuron pool is expanded in *Fzd3*-/- mice

Since the lateralization defect observed in *Fzd3*-/- mice was not a result of gross anatomical changes in the midbrain, we further studied the distribution of mdDA neurons using a marker for lateral mdDA neurons, Raldh1/Aldh1a (Aldehyde dehydrogenase family 1, subfamily a1) (Adh2) (Wallen et al, 1999; Jacobs et al, 2007). At E14.5, *Adh2* mRNA was detected within the more lateral aspects of the mdDA system (Fig. 5A, B). In E14.5 *Fzd3*-/- embryos, *Adh2* mRNA expression was still limited to the domain of TH expression and confined to more lateral neurons. However, expression of *Adh2* clearly marked the lateral expansion of mdDA neurons visualized by TH staining confirming the idea that the lateral mdDA neuron pool has expanded along the mediolateral axis in *Fzd3*-/- mice (Fig. 5C, D). Sagittal sections showed compact expression of *Adh2* at the mesodiencephalon flexure in *Fzd3*+/+ embryos (Fig. 5E, F). In *Fzd3*-/- embryos, *Adh2* expression had expanded caudally, consistent with the caudal expansion of the TH+ neuron population. The change of *Adh2* expression in relation to TH+ neuronal expression confirms that the lateral population of mdDA neurons is affected in *Fzd3*-/- mice.

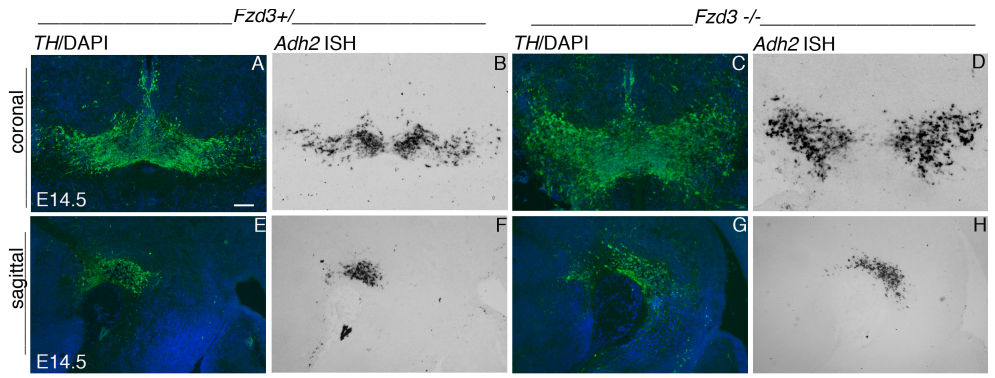

Figure 5. A lateral subpopulation of mdDA is disorganized in Fzd3 deficient mice.
Adh2 expression in *Fzd3*+/+ and *Fzd3*-/- mice in combination with tyrosine hydroxylase (TH) immunohistochemistry in coronal (A-D) and sagittal (E-H) sections. (A, B, E, F) In wt, *Adh2* mRNA is selectively expressed in lateral TH+ neurons. (C, D, G, H) However, this lateral set of neurons has expanded laterally and caudally in *Fzd3*-/- mice. Scale bar=40 mM (A-F).

Fzd3 deficient mice exhibit changes in the radial glia scaffold

How does Fzd3 regulate the mediolateral organization of the mdDA system? One possible mechanism is that Fzd3 regulates the migration of mdDA neurons from the ventricular zone to more ventral aspects of the midbrain. Interestingly, Fzd3 has been reported to control facial branchiomotor neuron migration (Vivancos et al, 2009). Therefore, we examined whether mdDA neuron migration was intact in *Fzd3*-/- mice. In wt mice, the

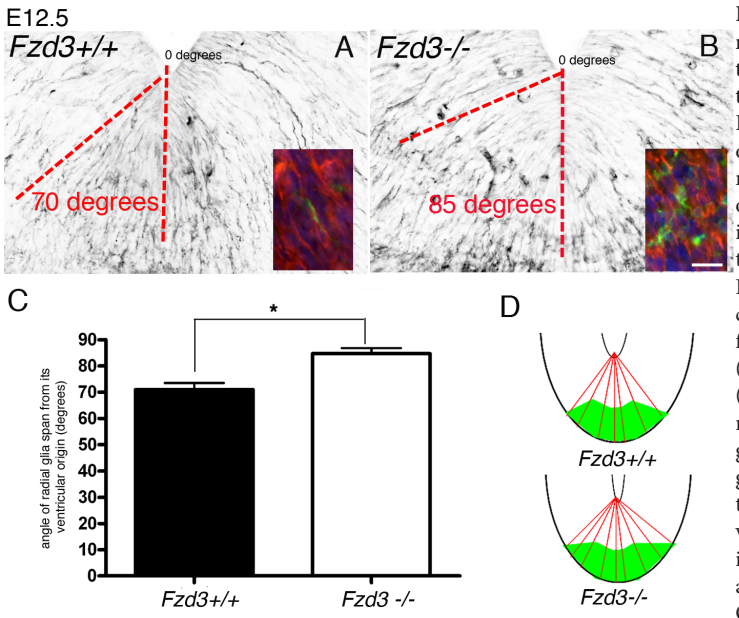

Figure 6. Fzd3 deficient mice exhibit changes in the radial glia scaffold of the midbrain.
Immunohistochemistry on coronal sections for nestin shows a widening of the radial glia scaffold in *Fzd3*-/- (B) compared to *Fzd3*+/+ embryos (A). Inserts in A and B show coronal sections stained for tyrosine hydroxylase (TH; green) and nestin (red) and show TH+ neurons migrating along radial glia fibers during early migration at E12.5. Note that the distance between individual radial glia fibers has increased in *Fzd3*-/- mice as compared to wt. (C) Quantification of the trajectory of individual radial glia fibers (for details see Materials and Methods. (D) Schematic indicating the observed defect. The distance between radial glia fibers is increased in *Fzd3*-/- embryos causing migrating mdDA neurons to reach more lateral aspects of the midbrain. *p < 0.05 , one-way ANOVA. Scale bar=40 mM (A, B), 160 mM (inserts).

first mdDA neurons begin to migrate from the ventricular zone around E11.5. The precise mode of migration of mdDA neurons is still unclear. Two putative models have been proposed, mdDA neurons may migrate radially along the radial glia only or they may undergo biphasic migration (Kawano et al, 1995). Both modes of migration require neurons to migrate ventrally along radial glia. This has been demonstrated by co-staining of TH with the radial glial marker, nestin (Ohyama et al, 1998). To examine whether mdDA neuron migration and radial glia are affected in *Fzd3*-/- mice, sections were subjected to co-immunohistochemistry for TH and nestin. In both *Fzd3*+/+ and *Fzd3*-/- embryos the organization of the nestin+ radial glia scaffold was intact with glial fibers descending from the ventricular zone. No clear changes in the number of radial glia cells or in the number of TH+ cells associated with nestin+ fibers were observed (data not shown).

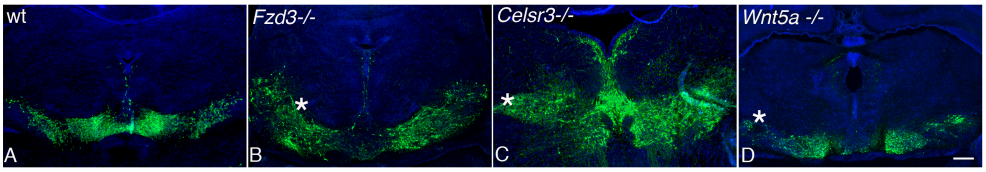

Figure 7. Celsr3 deficient mice display defects in the organization of the mdDA system similar to Fzd3 deficient mice
Immunohistochemistry for TH (in green) was performed on E17.5 coronal sections. (A-C) As compared to wt mice, mdDA neurons are laterally expanded in E17.5 *Fzd3*-/- and *Celsr3*-/- mice. (D) *Wnt5a*-/- mice show subtle laterization (asterisks). Scale bar=40 mM (A-D)

However, in *Fzd3*-/- embryos a widening of the radial glia scaffold was observed. Radial glia fibers initiated at comparable positions at the ventricular zone contacted more lateral parts of the ventral midbrain in *Fzd3*-/- as compared to *Fzd3*+/+ (Fig. 6A, B).
To quantify this observation, we measured the angle between the midline and the path of individual radial glia. This analysis showed a widening of the radial glia scaffold of approximately 15 degrees in *Fzd3*-/- compared to *Fzd3*+/+ (Fig. 6C). Since mdDA neurons utilize radial glia for their ventral migration, a widening of the radial glia scaffold may cause mdDA neurons to reach more lateral positions (Fig. 6D). This suggests that the lateralization defect observed in *Fzd3*-/- is a result of defects in the organization of radial glia.

Other PCP proteins also contribute to the cellular organization of the mdDA system. Fzd3 is a PCP receptor and the defects described above are classic PCP phenotypes. To determine if other PCP proteins are involved in the development of the mdDA system, we analyzed mouse lines deficient in other PCP proteins (Fig. 7).
Celsr3 (cadherin EGF LAG seven pass G-type receptor 3) is a transmembrane receptor that works together with Fzd3 in PCP signaling. Analysis of *Celsr3*-/- mice using TH immunohistochemistry at E17.5 revealed lateralization defects similar to those observed in *Fzd3*-/- embryos. This suggests that *Celsr3* and *Fzd3* cooperate to organize the mdDA neuron pool. We further examined the most likely ligand for PCP signaling, Wnt5a. A previous study reported a lateralization defect in *Wnt5a*-/- mice but analysis at E17.5 only showed a subtle lateralization of mdDA neurons. This suggests that the effect of *Wnt5a* deficiency may be compensated by other Wnts expressed in the mdDA system (Andersson et al, 2008). In all, laterization defects in the mdDA system are observed in *Celsr3* and *Wnt5a* deficient mice, suggesting that Fzd3, Celsr3 and Wnt5a may function together to organize the mdDA neuron pool.

Discussion

Wnt signaling has been implicated in several cellular processes during the development of the mdDA system, but little is known about the Wnt receptors involved. In this study, we identify a role for the Wnt receptor Fzd3 in regulating the cellular organization of the mdDA neuron pool. We show that *Fzd3* is expressed in the developing mdDA system and that its absence in mice causes a mediolateral and rostrocaudal expansion of the mdDA system. This abnormal organization of mdDA neurons in *Fzd3*-/- mice was independent of the general organization of the midbrain region. Moreover, similar organization defects were present in mice lacking the PCP receptor *Celsr3,* and to a lesser extent in *Wnt5a* mutant mice. Overall, our results indicate that PCP signaling is crucial for the proper cellular organization of the mdDA system.

Fzd3 controls the organization of the mdDA neuron pool

The present study reveals that in addition to mdDA axon guidance (Fenstermaker et al., 2010) Fzd3 is required for the cellular organization of the mdDA neuron pool. In *Fzd3* deficient mice, mdDA neurons expanded both laterally and caudally. Interestingly the A-P gradient of *Fzd3* expression in the midbrain is consistent with more pronounced

effects in the caudal mdDA system in the *Fzd3*-/- mice, where *Fzd3* was most intensively expressed. The observed defects gradually increased throughout development. At E11.5, subtle abnormalities were observed consisting of laterally protruding axons (Fenstermaker et al., 2010). By E14.5, mdDA neurons were prominently disorganized with neurons in regions lateral to the normal mdDA neuron pool and a caudal increase in the number of mdDA neurons. Similar defects were observed at E17.5. Interestingly, by using several molecular markers (Fgf8, Gbx2, FoxA2), we found that the overall patterning of the mesodiencephalon in relation to the TH+ mdDA neuron pool was intact in *Fzd3* mutant mice. Thus, the expansion of the mdDA neuron pool is not simply a result of changes in the organization or size of cellular compartments in the midbrain.

In contrast to the patterning of the midbrain region, defects were observed in the radial glia scaffold of the midbrain. The migration of mdDA neurons requires alignment with guiding fibers, such as radial glial fibers and tangential nerve fibers. Several papers suggest that mdDA migration is biphasic (Kawano et al, 1995; Shults et al, 1990; Ohyama et al., 1998). First, young mDA neurons from the ventricular surface migrate to the ventromedial mesodiencephalon along radial glial fibers. Second, they migrate laterally in the basal part of ventral mesodiencephalon along tangentially arranged nerve fibers. In *Fzd3*-/- mice the distance between individual radial glia fibers was increased (Fig. 7). Radial glia extending from a similar location in the ventricular zone of *Fzd3*+/+ and *Fzd3*-/- embryos were found to connect to a more lateral region in the ventral midbrain in *Fzd3*-/- mice as compared to control. The consequence of this abnormal projection of radial glia fibers maybe that mdDA neurons originating in the same region in *Fzd3*+/+ and *Fzd3*-/- mice will end up more laterally in *Fzd3*-/- mice.

The radial glia scaffold in the medial aspect of the midbrain appeared largely intact, providing a possible explanation for the absence of defects of more medial parts of the mdDA neuron pool in *Fzd3*-/- embryos.

This result indirectly confirms that mdDA neurons utilize radial migration to reach the ventral base of the midbrain. Whether lateral migration occurs normally in *Fzd3*-/- mice remains to be established. Our findings do however raise the question if biphasic migration is required for all mdDA neurons since the general organization of the medial mdDA population was intact. The broadening of the radial glia scaffold may be a direct effect of morphological defects in A-P and D-V axes commonly observed in PCP deficient mice (Wallingford and Harland, 2002). A broadening of the ventral mesencephalon may cause an increased spacing between individual radial glia fibers. If this hypothesis is true, other more lateral neuronal populations that utilize radial migration should also display laterization defects. Future work will address this hypothesis.

The organization of the mdDA neuron pool requires different PCP proteins

Fzd/PCP signaling is an evolutionary conserved mechanism and requires complex interactions between different PCP molecules (Montcouquiol et al, 2008). Among the 10 Fzd family members, Fzd 1, 2, 3, 6, 7 and 9 are expressed in the midbrain region. Fzd3 is most intensively expressed in the midbrain and Fzd6 has an exclusive caudal mdDA expression, which overlaps with Fzd3 expression (Fischer et al, 2007). Within the Fzd family of receptors only Fzd3 and Fzd6 have been implicated in PCP signaling. Fzd3/PCP is required for neural tube closure and planar orientation of hair bundles in auditory sensory

cells of mice (Wang et al, 2006). Fzd6 has been shown to have a role in PCP signaling in inner ear sensory hair cells (Wang et al, 2006). A recent study shows that *Fzd6* deficient mice display no significant defect in the mdDA system. However, *Fzd3-/-; Fzd6-/-* double mutant mice showed severe defects in the overall organization of the midbrain (Stuebner et al, 2010). This is perhaps due to the ability of Fzd3 to compensate for the absence of Fzd6.

Based on these and our own results it is tempting to speculate that within the Fzd family of receptors, Fzd3 is most crucial for mdDA morphogenesis and the organization of the midbrain. Our expression studies revealed that *Fzd3* was expressed both in and around mdDA neurons. PCP signaling requires communication between adjacent cells to coordinate cellular movement (Montcouquiol et al, 2006 and Strutt DI, 2001). For example, interaction between mdDA neurons and non-mdDA neurons (such as glia) is required for migration. Fzd3 expressed in mdDA neurons and adjacent cells could signal with other core PCP molecules (discussed below) to induce cytoskeleton changes inducing migration (see Figure 8a). In *Fzd3* deficient mice, radial migration seems intact, where mdDA neurons born in the ventricular zone do migrate to the ventral mesodiencephalon. However, the radial glia scaffold has broadened, explaining the more lateral final destination of mdDA neurons in *Fzd3-/-* mice.

To establish the role of Fzd/PCP in the organization of radial glia scaffold, further experiments are needed. Other core PCP molecules in mice are Vangl2 (Vang-like 2) and Celsr3. Like Fzd3, these molecules are transmembrane proteins. *Celsr3* deficient mice display a similar lateral expansion of TH+ neurons as observed in *Fzd3-/-* mice (Fig. 7). This indicates that Fzd3 and Celsr3 may signal in concert. Future studies are needed to establish whether the radial glia scaffold of *Celsr3-/-* mice is changed. How could Fzd3 and Celsr3 cooperate to regulate radial glia patterning? In the forebrain, axonal tracts in *Celsr3* and *Fzd3* deficient mice show major and comparable defects. Celsr3 is expressed on both neurons and guidepost cells and may promote adhesion thereby bringing together Fzd3 and Vangl2 to form heterophilic interactions required for proper PCP signaling (Zhou et al, 2008). It is unclear if similar mechanisms are involved in the organization of the mdDA system. To verify if this kind of PCP signaling is involved, studying the expression of Vangl2 in the mdDA system would be necessary. In addition immunohistochemistry with anti-TH to examine potential defects in the mdDA system of *Vangl2 -/-* mice will verify the involvement of PCP signaling.

The only known ligands for PCP signaling are Wnt proteins. Among the 19 members of the Wnt family, Wnt5a has been implicated in PCP signaling in facial branchiomotor neuron migration, stereocilia, cochlear extension, and neural tube closure (Vivancos et al, 2009). In the developing mdDA system, *Wnt5a* is expressed in characteristic gradient along the A-P axis (Andersson et al., 2008; Fenstermaker et al., 2010). The low rostral and high caudal mRNA expression of *Wnt5a* is reciprocal to the *Fzd3* expression pattern described in this study. The expression of Wnt5a suggests that it could be a ligand for Fzd3. The *Wnt5a-/-* mice studied by Andersson et al., 2008 also showed dis-morphogenesis of the ventral midbrain. These authors found that TH+ neurons were reduced along the rostrocaudal axis and that they were laterally expanded (Andersson et al., 2008, Figure 8). However, the organizational defect in *Wnt5a-/-* was transient and subtle compared to *Fzd3 -/-* phenotype. Based on *Wnt5a* expression and *Wnt5a-/-* defects

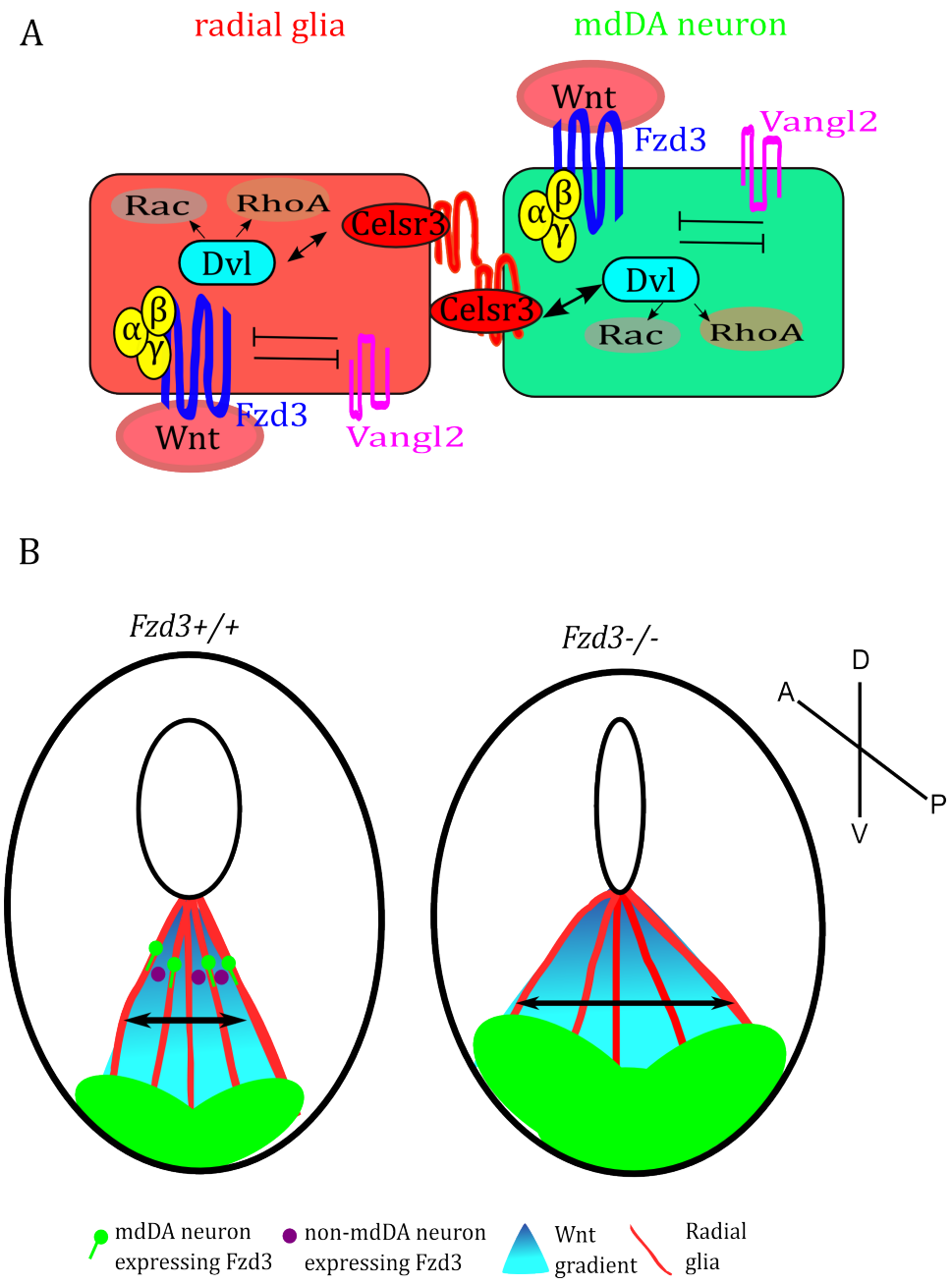

Figure 8: Overall proposed hypothesis of the role of Fzd3/PCP signaling in the organization of the mdDA neuron pool. (A). The core PCP molecules Fzd3, Celsr3 and Vangl2 are expressed in both mdDA neurons and radial glia. With Wnt activation Celsr3 may promote adhesion between mdDA neurons and radial glia and bring together Fzd3 and Vangl2 to form heterophilic interactions required for proper PCP signaling.(B) Schematics show coronal view of wt and *Fzd3-/-* phenotype. In wt, mdDA neurons expressing Fzd3 migrate along the tightly knitted radial glia scaffold in response to a Wnt gradient. In *Fzd3-/-* the radial glia scaffold has widened, resulting expanded localization of mdDA neurons at the ventral mesodiencephalon.

described in literature in addition to our findings, which show a subtle laterization of mdDA neurons (Fig. 7D), it seems that Wnt5a works together with Fzd3/PCP proteins in organizing the mdDA neuronal pool.

Acknowledgements
We would like to thank Marten Smidt for providing *Gbx2, Adh2* and *FoxA2 in situ* probe constructs and Horst Simon for the *Adh2 in situ* probe constructs. We are grateful to Rejji Kuruvilla and Jeremy Nathans for generously providing the *Fzd3* mutant mice. We thank Steven Stacker for providing the *Ryk* mutant mice and Andre Goffinet and Fadel Tisser for *Celsr3* mutant mice. The Netherlands Organization supported this work for Health Research and Development (ZonMW-VIDI and ZonMW-TOP), the International Parkinson Foundation, the Human Frontier Science Program (Career Development Awards), and the European Union (mdDANeurodev, FP7/2007-2011, Grant 222999) (to R.J.P.). This study was performed in part within the framework of Dutch Top Institute Pharma Project T5-207 (to R.J.P.).

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Chapter 5

A role for Wnt5a/Ryk signaling in the formation of mesostriatal circuitry?

Asheeta A. Prasad and R. Jeroen Pasterkamp

Abstract

Mesostriatal circuitry consists of mesodiencephalic dopaminergic (mdDA) axon projections from the substania nigra (SN) and ventral tegmental area (VTA) forming synapses with medium spiny neurons (MSNs) in the striatum and axons from MSNs projecting back to the SN. This circuitry is fundamental for motor control, motivation and reward seeking behavior and has been implicated in the pathology of various neurological disorders. Wnt5a and its receptor Ryk regulate the development of several axon tracts in the developing brain by inducing repulsive axon guidance. In this study, we explored the potential axon guidance role of Wnt5a and Ryk during the formation of mesostriatal circuits. We found that *Wnt5a* and *Ryk* are expressed during the formation of mesostriatal circuitry and that Wnt5a can elicit chemorepulsive effects on mdDA axons in culture. We previously showed that Fzd3 is involved in Wnt5a axon repulsion. Here we blocked Ryk signaling using an anti-Ryk antibody and found that Ryk mediates Wnt5a chemorepulsive responses in mdDA axons *in vitro*. Despite this robust chemorepulsive effect *in vitro,* no prominent defects were observed in mesostriatal or striatonigral axon projections in *Wnt5a-/-* or *Ryk-/-* mice. This suggests that the lack of Wnt5a and Ryk can be compensated by other molecular cues *in vivo*.

Introduction

Mesostriatal connectivity is fundamental for motor control, motivation and reward seeking behavior (Gerfen et al, 2002; Bjorklund and Dunnett 2007). Mesostriatal circuitry consists of connections between medium spiny neurons (MSNs) in the striatum and mesodiencephalic dopaminergic (mdDA) neurons in the midbrain. mdDA axons (located in the substantia nigra (SN) and ventral tegmental area (VTA)) project rostrally to form synapses in the striatum (Prasad et al., 2007;Van den Heuvel and Pasterkamp, 2008). Vice versa, a subset of MSNs, i.e. striatonigral neurons, projects their axons to the SN (Lobo et al, 2007). Both tracts run longitudinally along the A-P axis of the telencephalon in opposite directions. For the proper development of these tracts, several axon guidance molecules must work together to ensure that axons grow in the correct A-P orientation, form fasciculated axon bundles and innervate their distant targets. Several axon guidance cues are known to contribute to mdDA pathway development (see chapter 1 and 2 for details). In contrast, molecular cues regulating the growth and guidance of MSN axons remain unknown.

Wnt5a is a member of a large family of secreted proteins with 19 members in mammalian species (Miller JR, 2002). Wnts exert a wide variety of biological functions. For example, phenotypes observed in *Wnt5a-/-* mice include shortened limbs, facial abnormalities and prenatal lethality (Yamaguchi et al, 1999). In the central nervous system (CNS), Wnt5a has been shown to act as a chemorepulsive cue for callosal and corticospinal axons (Keeble et al, 2006; Liu et al, 2008). Wnt5a also influences the migration of craniofacial neurons, the morphogenesis of the mdDA neuron pool and synapse formation in hippocampal cultures (Vivancos et al, 2009; Andersson et al, 2008; Castelo-Branco et al, 2005, Davis et al, 2008). To control these diverse cellular functions Wnt5a utilizes Frizzled, Ror and Ryk receptors (Mikels et al, 2009; Li et al, 2010; Sato et al, 2010).

Ryk is a tyrosine kinase receptor and Wnts are the only known ligands for Ryk. Ryk/Wnt interactions can induce both canonical and non-canonical signaling (Lu et al, 2004). *Ryk* mutant mice phenocopy *Wnt5a* mutants for craniofacial and limb defects (Halford et al, 2000). However, the best characterized role for Ryk/Wnt signaling is in axon guidance. Retinal ganglion cell (RGC) repulsion is mediated by Wnt3/Ryk signaling and corticospinal tract axons expressing Ryk are repelled by Wnt1 and Wnt5a *in vivo* (Schmitt et al, 2006, Liu et al, 2008). Transgenic mice expressing *Ryk* siRNA exhibit defects in axon guidance (Lu et al, 2004). Furthermore, Ryk is required for neurite outgrowth induced by Wnt3a (Lu et al, 2004). In the corpus callosum, Wnt5a is expressed adjacent to the midline and callosal axons expressing Ryk cross the midline and are repelled to the contralateral side by Wnt5a (Keeble et al, 2006). The Ryk intracellular domain can be cleaved in response to Wnt3. Following cleavage, the Ryk cytoplasmic domain translocates to the nucleus, inducing neuronal differentiation (Lyu et al, 2008).

In a previous study, we have shown that Wnt5a/Fzd3 signaling is required for the formation of mdDA pathways (Fenstermaker et al, 2010). We found that depending on their location or developmental stage mdDA neurons and their axons can respond differentially to the same cue, Wnt5a. This suggests that multiple different receptor and signaling proteins are involved in mediating the responses to Wnt5a in mdDA system.

Here we examined whether Ryk is involved in Wnt5a-mediated axon guidance in mes-

ostriatal circuitry. We found that *Wnt5a* and *Ryk* are expressed during the development of mesostriatal circuitry. *Wnt5a* expression is present in mdDA neurons, but not in the striatum. *Ryk* was expressed in a high rostral to low caudal gradient in the developing forebrain. Wnt5a induced robust repulsion of mdDA axons in culture, an effect that could be blocked by anti-Ryk antibodies. This demonstrates that Wnt5a/Ryk signaling is likely to occur in the mdDA system. We next examined mesostriatal circuitry in both *Wnt5a* and *Ryk* deficient mice at E17.5, a developmental timepoint when mdDA and striatonigral axons have reached the striatum and SN, respectively. Surprisingly, no prominent axon defects were detected in the mdDA and mesostriatal pathway in *Wnt5a* -/- and *Ryk* -/- mice. This suggests that the loss of Wnt5a and Ryk may be compensated for by other molecular cues, including other Wnt proteins and Wnt receptors.

Materials and Methods

Animals

All animal use and care was in accordance with institutional guidelines. C57BL/6 mice were obtained from Charles River. *Wnt5a* mutant mice were purchased from the Jackson Laboratories. *Ryk* mutant mice a kind gift of Steven Stacker [(Ludwig Institute for Cancer Research, Australia (Halford et al., 2000)]. Timed-pregnant mice were killed by means of cervical dislocation. The morning on which a vaginal plug was detected was considered embryonic day 0.5 (E0.5).

In situ hybridization

E17.5 mouse brains were collected and immediately frozen on powdered dry ice. Brains were cryosectioned (14 μm) and mounted on Superfrost Plus slides (Thermo Fisher Scientific), air-dried and stored desiccated at -80^{0}C. For non-radioactive *in situ* hybridization, sections were washed in sterile phosphate buffered saline (PBS), pH 7.4, three times for 5 min each and then acetylated for 10 min in 1% triethanolamine solution and acetic anhydride dissolved in deionised water. Sections were washed with PBS three times for 5 min and then pre-hybridized with hybridization solution (2x standard saline citrate (SSC) buffer, 50% formamide, 5x Denhardt's solution, 250 μg/ml bakers yeast tRNA, 500 ug/ml sonificated salmon sperm DNA) for 2hrs at RT. Next, digoxigenin (DIG)-labeled riboprobe in a total volume of 150 μl of hybridization solution and at a concentration of 400 ng/ml was added onto each slides. Slides were incubated in tightly sealed containers overnight at 63^{0}C in a humidified incubator. Specific DIG-labeled *in situ* probes were generated with the following primers: *Wnt5a* Forward: AAT TCG TGG TGT GAA TGA ACT, *Wnt5a* Reverse: ACT CCC GGG CTT AAT ATT CC, *Ryk* Forward: ATT TCT GCT CAA GGG GAG GT, *Ryk* Reverse: TTG GGT GTG TCA GCT CTC AG. The following day sections were washed in 2x SSC buffer for 5 min at 63^{0}C followed by 2hr incubation in 0.2x SSC at 63^{0}C. Sections were adjusted to RT in 0.2x SSC for 5 min. Sections were then incubated with blocking buffer containing 10% fetal calf serum (FCS) in Buffer A (100 mM Tris-HCL, pH 7.4, 150 mM NaCl) for 1hr at RT, followed by incubation with anti-DIG Fab fragment conjugated to alkaline phosphatase (Boehringer) in Buffer A containing 1% FCS for 2hr at RT (1:2500). Then, sections were washed three

times for 5 min in Buffer A and once for 5 min in Buffer B (100mM NaCl, 5mM $MgCl_2$, 100 mM Tris-HCL, pH 9.5). The colouring reagent, nitroblue tetrazolium chloride with 5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP, Sigma), and levamisole (2.4 mg/ml) were dissolved in buffer B and added to slides for overnight incubation at RT. The following day, sections were rinsed in 10 mM TE buffer (Tris-HCL, pH 8.0, 5mM EDTA) and dehydrated in an increasing concentration of ethanol (30%-100%), cleared in xylene and embedded in entellan.

Immunohistochemistry

Embryos were collected from timed-pregnant mice and incubated in 4% PFA for 1 hr at RT, followed by immersion in 30% sucrose in PBS. Brains were frozen on powdered dry ice, cryosectioned (14 µm), mounted on Superfrost Plus slides (Thermo Fisher Scientific), air-dried, and stored desiccated at -80^{0}C. For immunohistochemistry, sections were washed in PBS, pH 7.4. Non-specific antigens were blocked with blocking solution [3% Normal Goat Serum, 1% Normal Horse Serum, 1% Normal Donkey Serum, 1% Bovine Serum Albumin, 1% Glycine, 0.1% Lysine, 0.4% Triton-X-100 in PBS] for 30 minutes at RT. Primary antibodies were incubated overnight in blocking buffer at 4^{0}C. The following primary antibodies were used: rabbit anti-TH (1:1000, Pel-Freeze) and rabbit anti-DARRP32 (1:500, Santa Cruz). Next, sections were washed in PBS and incubated for 1 hr at RT with the appropriate AlexaFluor-labeled secondary antibodies. Sections were washed in PBS, counterstained with fluorescent DAPI (Sigma), washed extensively and embedded in Mowiol (Sigma). Staining was visualized using a Zeiss Axioskop 2 microscope.

TH explant assays

Three-dimensional collagen matrix assays using mdDA explants were performed as described previously (Kolk et al., 2009; chapter 3). mdDA explants were rapidly dissected from E14.5 C57BL/6 embryos and embedded in close proximity to aggregates of HEK293 cells (~300 µm apart) transiently transfected with an expression vector for Wnt5a or mock-transfected with a control vector. Explants were cultured for 3 days, fixed and co-immunostained using rabbit anti-TH (1:1000; Pel-Freeze) and mouse anti-ßIII-tubulin (1:3000; Sigma) antibodies to stain mdDA and all neurites emerging from the explants, respectively. For Ryk blocking experiments, E14.5 mdDA explants were cultured with Wnt5a aggregates in the presence of either IgG goat antibodies (4 µg/ml; Calbiochem), as a control, or Ryk antibody (25 µg/ml, a kind gift of Yimin Zou, (UCSD) (Schmitt et al, 2006)). For quantification of explant assays, the length of the 20 longest TH-positive neurites was measured in both the proximal and distal quadrants of the explant cultures using OpenLab software (Improvision). The average value of the 20 proximal and distal neurites was used to determine the proximal/distal ratio (P/D ratio) per explant. Data were statistically analyzed by one-way ANOVA (α=5%) and expressed as means ±SEM.

Results

***Wnt5a* and *Ryk* are expressed during the development of mesostriatal connectivity.**
The development of mesostriatal circuitry requires the pathfinding of striatal and mdDA axons between the striatum and the midbrain. Since both populations of axons maneuver through the same regions in the forebrain, we hypothesized that they are affected by a similar set of guidance cues expressed along their trajectories.
Here we focused on Wnt5a and its receptor Ryk, which have been implicated in axon outgrowth and repulsion in different regions of the nervous system (e.g. Keeble et al, 2006; Schmitt et al, 2006). To examine whether these molecules also play a role in axon guidance during the formation of connections between mdDA neurons and MSNs, we first assessed *Wnt5a* and *Ryk* mRNA expression in the midbrain and striatum and along the nigrostriatal and mesostriatal trajectory. As reported previously, at E14.5 Wnt5a was expressed at the mesencephalic flexure (Andersson et al, 2008) but no or faint Wnt5a expression was found in the striatum. However, Wnt5a was expressed in (sub)ventricular regions (Fig. 1A). In the mdDA region, *Wnt5a* signals co-localized with tryrosine hydroxlyase (TH) expression suggesting that mdDA neurons express Wnt5a, as suggested previously (Andersson et al, 2008) (Fig. 1C-E). A similar expression pattern was observed at E17.5 (data not shown). At E14.5 and E17.5, *Ryk* was expressed throughout the forebrain in a high rostral to low caudal gradient along the A-P axis. Intense Ryk expression was detected in the forebrain and in the striatum (Fig. 1B). Ryk was also expressed

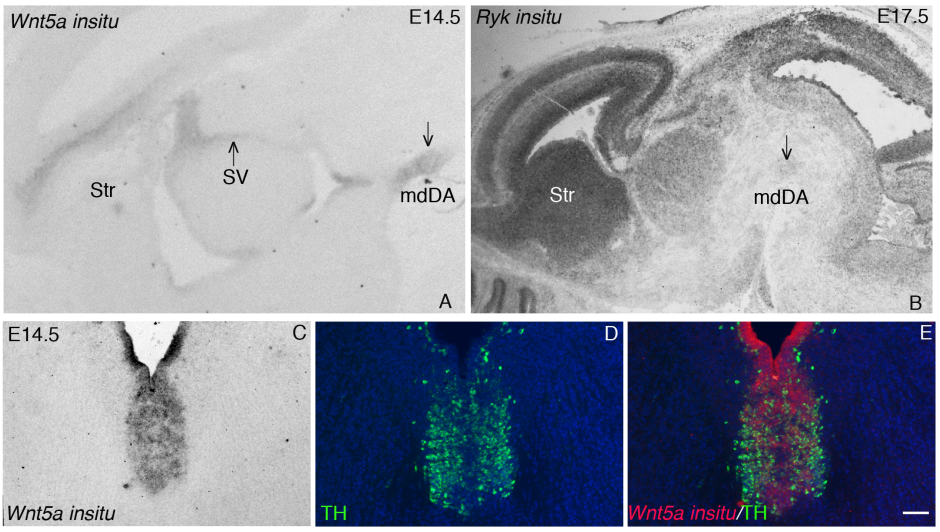

Figure 1. *Wnt5a* and *Ryk* are expressed during the development of mesostriatal circuitry.
(A) *In situ* hybridization for *Wnt5a* at E14.5 sagittal section shows *Wnt5a* is expressed distinctly on the mesencephalic flexure indicated by arrow and very weak expression in the subventricular zone (SV) indicated by arrow. (B) *In situ* hybridization at E17.5 sagittal section shows *Ryk* mRNA is expressed in the whole brain in a decreasing gradient along the A-P axis. *Ryk* is also expressed at the mdDA region indicated by arrow. (C-E) *In situ* hybridization for *Wnt5a* in combination with immunohistochemistry for tyrosine hydroxylase (TH) in green on coronal cryosections at E14.5. The *Wnt5a in situ* signal has been pseudo-colored (red) to show overlap of *Wnt5a* and TH expression. Scale bar=5μM (A), 20 μM (B), 40 μM (C-E)

in the mdDA region, albeit at lower levels (Fig. 1B, arrow). Co-localization experiments showed that Ryk signals were detected in a subset of TH-positive mdDA neurons (data not shown).

Wnt5a chemorepulsion is mediated by Ryk *in vitro*

The expression of Ryk in MSNs and mdDA neurons and of Wnt5a in the forebrain and midbrain supported a role for Wnt5a/Ryk signaling in striatonigral and nigrostriatal pathway development. Our previous work has shown that Wnt5a is highly potent repellent for mdDA axons via the Fzd3 receptor (Fenstermaker et al, 2010). However, several lines of evidence suggest that other receptor proteins cooperate with Fzd3 in mediating Wnt5a responses (Fenstermaker et al., 2010). Because of the prominent role of Ryk in axon guidance (e.g. Keeble et al, 2006 and Schmidt et al, 2006) and the expression of *Ryk* in the midbrain, we determined whether Ryk is also involved in mediating Wnt5a repulsion in mdDA axons. MdDA explants were co-cultured adjacent to control or *Wnt5a* secreting cells in the presence of either IgG or anti-Ryk antibodies. The anti-Ryk antibody is a polyclonal antibody directed against the Ryk ectodomain (Schmitt et al, 2006). mdDA axons displayed a radial pattern of outgrowth in the presence of cell aggregates transfected with empty vector (Fig. 2a). IgG antibodies did not affect this radial growth (data not shown). In contrast, mdDA axons were strongly repelled by Wnt5a secreting cells in the presence of IgG control antibodies (Fig. 2B). Intriguingly, this repulsive effect of Wnt5a was completely lost in the presence of anti-Ryk antibodies (Fig. 2D).

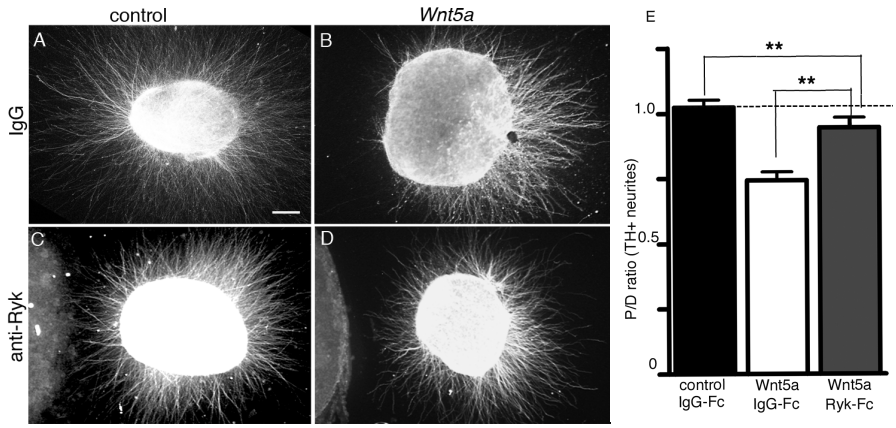

Figure 2. Wnt5a chemorepulsion of mdDA axons is mediated by Ryk.
mdDA explants dissected from E14.5 wt embryos were cultured adjacent to HEK293 cells secreting control protein (A; n=9) or Wnt5a (B; n=9) in the presence of control IgG or anti-Ryk antibody. Explants were cultured for 3 days and immunostained with anti-Tyrosine hydroxylase. Quantification of the length of TH-positive neurites in the proximal (P) and distal (D) quadrants of culture assays is shown (E). The graph shows average P/D ratio ±SEM, **p<0.05, one-way Anova. Note that, *Wnt5a* repulsion of TH-positive axons is blocked by anti-Ryk antibodies. Scale bar (A-D) =80μm

In all, these data show that Ryk is required for mediating Wnt5a repulsion in mdDA axons *in vitro.* Further studies are needed to assess whether Wnt5a also functions as a repellent for striatal axons in a Ryk-dependent manner.

mdDA axon pathways in the telencephalon are intact in *Wnt5a* and *Ryk* deficient mice

Based on our finding that Ryk antibodies are capable of blocking Wnt5a chemo-repulsion *in vitro*, we predicted a role for Wnt5a/Ryk in mdDA axonal pathway development. Therefore, the trajectory of mdDA axons in the telencephalon and the innervation of the striatum by mdDA axons were examined in *Wnt5a*-/- and *Ryk* -/- mice using immunohistochemistry for tyrosine hydroxylase (TH) (Fig. 3). No defects were observed in the morphology of mdDA pathways in the forebrain or in the innervation of the striatum in *Wnt5a*-/- and *Ryk*-/- mice (Fig. 3). Due to the gross morphological defects present in *Wnt5a*-/- mice, the morphology of striatum was slightly altered. Nevertheless, prominent mdDA innervation of the striatum was observed in *Wnt5a* deficient mice.

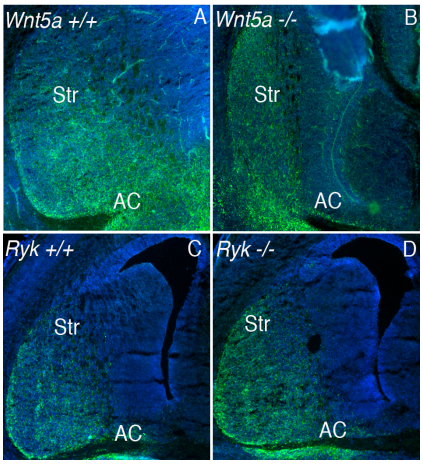

Figure 3. *Wnt5a* and *Ryk* deficient mice show no defects in the mdDA innervation of the striatum.
Coronal sections were immunostained with anti-Tyrosine hydroxylase antibody (in green) to visualize striatal innervation by mdDA axons in E17.5 *Wnt5a* wt and *Wnt5a*-/- (A,B) and *Ryk* wt and *Ryk*-/- mice . Sections were counterstained with DAPI and the striatum (Str), anterior commissure (AC) and lateral ventricle (LV) were used as hallmarks. Note, that innervation of the striatum by mdDA axons appears normal in *Wnt5a*-/- and *Ryk*-/- mice. Scale bar = 40µm

Striatal pathways are intact in *Wnt5a* and *Ryk* deficient mice

Striatonigral projections emerge from MSNs in the striatum and synapse on neurons in the substania nigra (SN). Since no prominent defects were observed in mdDA axon projections in *Wnt5a* and *Ryk* mutant mice, we wondered whether Wnt5a/Ryk signaling was involved in the formation of the striatonigral tract instead. Ryk expression was especially intense in the telencephalon (including the striatum). We hypothesized that MSNs use the Ryk receptor for their navigation to and innervation of the midbrain by detecting Wnt5a. DARPP32 (D32) was employed as a marker for all striatal neurons and axons to analyze the development of the striatonigral tract in *Wnt5a* and *Ryk* deficient mice. In wildtype (wt) embryos, D32-positive striatal neurons were either

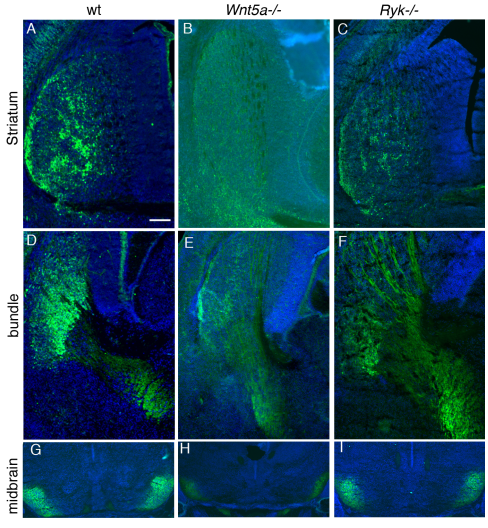

Figure 4. *Wnt5a* deficient mice show innervation of the midbrain by striatal axons
Immunohistochemistry for Darpp32 (D32, in green) was used to analyze striatal neurons and axons in E17.5 wt (A, D, G), *Wnt5a*-/- (B, E, H) and *Ryk*-/- (C, F, I) E17.5 mice at level of the striatum (A-C), the striatonigral bundle between the striatum and the midbrain (D-F), and in the midbrain (G-I). Sections were counterstained with DAPI (in blue). Scale bar= 40µm

accumulating in patches or spread around patches in the striatum. As striatal axons exit the striatum, they fasciculate into a tight bundle that travels towards the midbrain. At the midbrain, these axons form synapses in the SN (Fig. 4A, D, G). We examined the developing striatonigral tract at three different levels in *Wnt5a* and *Ryk*-/- mice; the striatum, midway and at the level of the SN. *Wnt5a*-/- mice display severe morphogenetic defects (Yamaguchi et al, 2009). In *Wnt5a*-/- brain, the lateral ventricle was present but closed and the anterior commissure was abnormally curved. Striatal neurons were present in *Wnt5a*-/- mice but D32-positive MSNs did not display the characteristic distribution in patches. The striatonigral bundle was intact. Expression of D32 in the midbrain of Wnt5a-/- mice was reduced compared to control indicating reduction innervation of the midbrain by striatal axons (Fig. 4B, E, H). In contrast to *Wnt5a*-/- mice, no defects were observed in the distribution of MSNs, the striatonigral tract or innervation of the midbrain in *Ryk*-/- mice (Fig. 4C, F, I).

The organization of the mdDA neuron pool is intact in *Wnt5a* and *Ryk* deficient mice

In a previous study, Andersson et al. (2008) reported that *Wnt5a*-/- mice show a lateral expansion of the mdDA neuron pool suggesting that Wnt5a regulates midbrain morphogenesis. In addition, we showed that *Fzd3* deficiency results in an abnormal organization of the mdDA neuron pool (chapter 3). Here, we attempted to confirm this role of Wnt5a and establish a role for Ryk in the regulation of the organization of the mdDA system. The mediolateral organization of the mdDA neuron pool was examined at different rostrocadaul levels in *Wnt5a* and *Ryk* mutant mice using TH as a marker (Fig. 5).

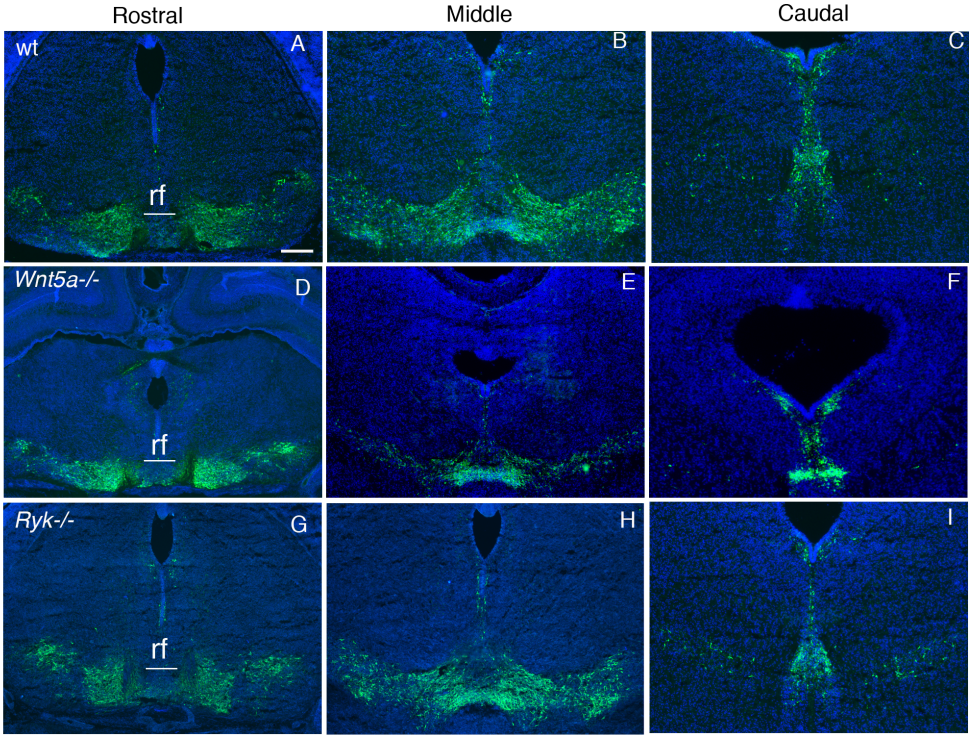

Figure 5. *Wnt5a* and *Ryk* deficient mice show no significant defect in the organization of the mdDA neuron pool.
Coronal sections were immunostained with anti-Tyrosine hydroxylase antibody (in green) and counterstained with DAPI (in blue) to analyze the organization of the mdDA neuron pool in wt (A-C), *Wnt5a*-/- (D-F) and *Ryk*-/- mice (G-I). Sections at different rostrocaudal levels are shown. The mdDA region is subsectioned into rostral, middle and caudal mdDA Note that no obvious defects are observed in *Wnt5a*-/- and *Ryk*-/- mutant mice. Rf, Retrofasciculus tract Scale bar = 40μm

At all rostro-cadaul levels examined, wt, E17.5 *Wnt5a*-/- and *Ryk*-/- mice displayed a similar mediolateral organization of mdDA neurons. The only defect observed was a decrease in the density of mdDA neurons in "middle" sections, at the level of the SN, in *Wnt5a*-/- mice. These results argue against a prominent role for Wnt5a and Ryk in the mediolateral organization of the mdDA system.

Discussion

Mesostriatal circuitry connects the striatum and the SN. Axons emerging from the striatum project to the SN running parallel, but in an opposite direction, to mdDA axons targeting the striatum. Defects in these trajectories underlie various neurological diseases including Huntington's and Parkinson's disease. Further insight into the mechanisms that control the formation and maintenance of mesostriatal connectivity will help to further our understanding of these disorders and may provide targets for therapeutic intervention. Our study shows that mdDA axons are responsive to Wnt5a repulsion in vitro and that these axons use Ryk, in addition to Fzd3 (Fenstermaker et al., 2010), to detect Wnt5a. Expression studies showed that *Wnt5*a is expressed in the TH+ mdDA system and along the ventricles of the telencephalon. *Ryk* was also expressed in the midbrain region but even more prominently in the striatum, which did not display *Wnt5a* expression. Based on these observations, we expected to find significant defects in the formation of mesostriatal and striatonigral tracts in *Wnt5a* and *Ryk* deficient mice. However, no defects were observed in striatal and mdDA projections in E17.5 *Wnt5a* and *Ryk* deficient embryos. Within the mdDA system, Wnt5a is required for the rostrally directed growth of mdDA axons in the midbrain. In the absence of *Wnt5a*, mdDA axons were found to project caudally as well at E12.5, but this defect was not detected anymore by E17.5 (Fenstermaker et al, 2010). *Wnt5a* -/- have also been reported to display midbrain morphogenesis defects and an expansion of mdDA subpopulations. Both of these phenotypes are also transient (Andersson et al, 2009). It is therefore possible that defects in mesostriatal connectivity in *Wnt5a* and *Ryk*-/- are only detectable before E17.5. In conflict with our findings, Blakely et al, (2011) recently reported an increase in the innervation of the striatum by mdDA axons in *Wnt5a*-/- mice. However, *Wnt5a*-/- embryos show severe defects in the overall morphology of the brain (Yamaguchi et al., 1999). This altered morphology can contribute to changes in the spatial arrangement and density of axons within a specific structure. Here, we for example report that the overall size of the striatum is decreased in *Wnt5a*-/- mice. Therefore, the defects reported by Blakely et al, (2011) may reflect a normal number of axons innervating a relatively smaller structure.

Ryk is required for Wnt5a repulsion

Wnt5a/Ryk signaling has been implicated in the development of several axonal tracts. Since Ryk was expressed in the midbrain region, we wanted to explore if Wnt5a/Ryk signaling is also required for the development of mdDA axon tracts. As reported previously, Wnt5a repelled axons emerging from E14.5 mdDA explants. This repulsion could be blocked by anti-Ryk antibodies indicating a role for Ryk in Wnt5a repulsion on mdDA axons. A role as a Wnt5a receptor for Ryk has previously been shown in the corpus callosum and the corticospinal tract (Keeble et al, 2005; Schmidt et al 2006). It was previously shown that Fzd3 is also required for mediating the repulsive effects of Wnt5a in culture (Fenstermaker et al., 2010) suggesting that Ryk and Fzd3 cooperate to form Wnt5a receptors. Fzd and Ryk interactions have been shown using immunoprecipitation approaches, where Ryk binds to the cysteine rich domain (CRD) of Fzd8 (Lu et al, 2004). However, it is unknown whether Ryk cooperates with Fzd3 in other axonal subpopulations. The absence of defects in mdDA projections in *Ryk* deficient mice may be explained by compensation by other Wnt receptors such as Lrp5/6 or Ror. The phenotypes observed in *Ror-/-* mice are similar to *Wnt5a-/-* phenotypes, e.g. shortened limbs and facial abnormalities (Oishi et al, 2003). In addition Wnt/Ror signaling is involved in neurite outgrowth in *Caenorhabditis elegans* (Kennerdell et al, 2009 and Song et al, 2010). Examination of Ror expression patterns and analysis of *Ror-/-* mice will help to establish their (compensatory) role in mesostriatal development.

Ryk mRNA is robustly expressed in the striatum but no abnormalities were observed in nigrostriatal connections in *Ryk-/-* mice. One explanation for this lack of a phenotype is that Ryk is involved in the development of other neuronal populations in the striatum such as the striatopallidal MSNs. It is also possible that Ryk regulates functions other than axon guidance during striatal development. For example, Ryk was recently shown to control specification of GABAergic neurons in the telencephalon (Zhong et al, 2011). It is interesting to note that in *Wnt5a-/-* mice no clear D32 striatal patches were present at E17.5 and that innervation of the midbrain by nigrostriatal axons was decreased. It is possible that that fewer striatal neurons are generated in *Wnt5a-/-* mice leading to reduction in the number of axonal projections. Future studies are needed to establish the mechanisms that underlie these defects and to identify the receptor proteins involved.

Overall this studies shows that Wnt5a functions as an axonal repellent for mdDA axons using Ryk as a receptor. The lack of strong defects in reciprocal mesostriatal connections in *Wnt5a-/-* and *Ryk-/-* mice indicates that other Wnts and Wnt receptors can compensate for the lack of Wnt5a and Ryk. Further studies are needed to establish which other Wnts and Wnt receptors contribute to the wiring of circuitry between the striatum and mdDA system.

Acknowledgements

We are grateful to Yimin Zou for generously providing the Ryk antibody. This work supported by the Netherlands Organization for Health Research and Development (ZonMW-VIDI and ZonMW-TOP), the International Parkinson Foundation, the Human Frontier Science Program (Career Development Awards), and the European Union (mdDANeurodev, FP7/2007-2011, Grant 222999) (to R.J.P.). This study was

performed in part within the framework of Dutch Top Institute Pharma Project T5-207 (to R.J.P.).

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Chapter 6

Frizzled3 is required for the formation of striatonigral and striatopallidal axon tracts

Asheeta A. Prasad, Francesca Morello, Youri Adolfs, R. Jeroen Pasterkamp

Manuscript under preparation

Abstract

The striatum is predominantly composed of GABAergic medium spiny neurons (MSNs). These neurons form two distinct efferent axon pathways, the striatopallidal (SP, indirect) and the striatonigral (SN, direct) tracts. These pathways are crucial for motor control, motivation and cognition functions. However, the molecular mechanisms controlling their formation and maintenance remain largely unknown. In this report, we used *D2-EGFP* and *M4-EGFP* BAC transgenic mice to examine the trajectory of SP and SN axons in the telencephalon throughout embryonic and early postnatal development. In addition, the expression of all members of the Frizzled family of receptors (Fzd1-10) was quantified in FACS purified populations of SP and SN neurons. Fzd3 and Fzd6, both previously implicated in axon tract development, were expressed in SP and SN neurons. Using different approaches, including the generation and analysis of *Fzd3-/-; D2-EFGP* and *Fzd3-/-; M4-EFGP* mice, defects in SP and SN neurons and their axonal projections were studied. *Fzd3-/-* mice showed major defects in the development of both SP and SN axon tracts with the majority of axons stalling at the globus pallidus (GP) and failing to innervate this intermediate target or extending to more distant targets. Overall our findings demonstrate that SP and SN axons require Fzd3 to form their highly specific axonal pathways. Further studies are needed to unveil molecular and cellular changes that prevent striatal axons from entering the GP in the absence of Fzd3.

Introduction

The striatum is mainly composed of GABAergic medium spiny neurons (MSNs). There are two types of MSNs, striatopallidal (SP) and striatonigral (SN) neurons, which have a different molecular profile, function and distinct axon projections. In the striatum, SP and SN neurons are mosaically distributed and either clustered together in "patches" or dispersed around these patches in a structure known as the "matrix" (Gerfen, 1992). SP neurons exclusively express dopamine receptor D1, muscarinic receptor M4 and tachykinin 1. Striatopallidal neurons express preproenkephalin 1, dopamine receptor D2 and adenosine receptor A2a (Lobo et al, 2007).

SP and SN axons form two distinct pathways. The SP tract, also known as the indirect pathway, carries axons to the globus pallidus (GP), which then forms connections to the subthalamic nucleus (STN). STN axons project to the substantia nigra pars reticulata (SNr). SN axons project directly from the striatum through the GP to the SNr (forming the direct pathway). The pathways work antagonistically, i.e. the direct pathway promotes movement and the indirect pathway inhibits movement thereby exerting a well-balanced control over movement (Graybiel, 2000). In addition to motor control, MSN connectivity also influences motivation and has been implicated in Huntington's disease, Parkinson's disease, drug addiction, depression and schizophrenia (Graybiel et al, 2008; Malenka et al, 2008; Baunez and Gubellini, 2010). However, although MSN neurons and their axonal projections subserve crucial physiological functions and are affected in several neural disorders, the molecular mechanisms that control their development and maintenance remain poorly characterized.

The recent generation of bacterial artificial chromosome (BAC) transgenic mice, which can be used to label specific cell types and their axonal projections (Gong et al, 2005), has provided a powerful method to visualize and isolate specific cell types. Using the exclusive gene expression profiles of SP and SN neurons, BAC transgenic mice with the enhanced green fluorescent protein (EGFP) gene inserted in the D2 or M4 loci have been created (Lobo et al, 2006). In *D2-EGFP* and *M4-EGFP* mice, SP and SN neurons, respectively, are labeled. These BAC transgenic reporter mice allow the purification of specific MSN subpopulations by fluorescence activated cell sorting (FACS) (Lobo et al, 2007).

In this study, we used *D2-EGFP* and *M4-EGFP* mice to examine the trajectory of SP and SN axons at several developmental stages. In addition, pure populations of SP and SN neurons were isolated use FACS to profile the gene expression patterns of all Frizzled family members. Both Fzd2 and Fzd3 were expressed in SP and SN neurons. Analysis of *Fzd3* deficient mice revealed that SP and SN neurons require Fzd3 to enter and traverse the GP. Our findings are the first to demonstrate that during specific parts of their trajectories SP and SN axons use common molecular cues for navigating (intermediate) target structures such as the GP.

Methods

Animals
All animal use and care were in accordance with institutional guidelines. Timed-pregnant mice were killed by means of cervical dislocation. The morning on which a vaginal plug was detected was considered embryonic day 0.5 (E0.5). *Drd2-EGFP* and *Chrm4-EGFP* mice on the FVB/N inbred background were obtained from MMRRC (www.gensat.org). These mice were bred with C57BL/6 mice obtained from Charles River. *Fzd3* heterozygous mice were obtained from Jeremy Nathans (Johns Hopkins University School of Medicine, Baltimore, USA (Wang et al., 2002)). *Drd2 (D2)-EGFP-Fzd3wt* and *D2-EGFP-Fzd3-/-* and *Chrm4(M4)-EGFP-Fzd3wt and M4-EGFP-Fzd3-/-* were generated by breeding *Fzd3* heterozygous mice with *D2-EGFP* and *M4-EGFP* mice.

FACS
The striatum was dissected from D2-EGFP and M4-EGFP brains at E15.5 and E17.5. Striatal tissue was dissociated using the Papain Dissociation System (Worthington) as previously described (Lobo et al, 2006). Cells were resuspended in L15-CO_2 without phenol and with 25-μg ml^{-1} DNase, followed by filtration through a 70-μm mesh. Cells were treated with propidium iodide (20 μg ml^{-1}) to label dead cells. Cells were sorted in a Cytopeia Infux cell sorter (528/38 filter for EGFP fluorescence). Approximately 70,000–150,000 striatal MSNs were obtained in each run from D2- and M4-positive embryos. GFP+ cells were collected in lysis buffer (Qiagen).

qPCR
Total RNA extraction was performed with the RNA Micro isolation kit (Qiagen) following the manufacturer's instructions. RNA quality and relative concentration were confirmed on the Bioanalyzer Nanochip (Agilent) and spectrophotometer. mRNA was amplified from 70 ng of total RNA by cDNA synthesis with oligo(dT) double-anchored primers, followed by *in vitro* transcription using a T7 RNA polymerase kit (Ambion) as described previously. The qRT-PCR reaction was performed in a 10 μl reaction solution using Roche SYBR Green I (LC-FastStart DNA MasterPlus) and 0.5 uM of each primer. Primer sequences for *Fzd1-10* and *Gapdh* were obtained from Shah et al. (2009). *D2* (Paula et al, 2008), *M4* (Wang and Seed, 2003) and *Hprt1* primers from QuantiTect primer Assay. The reaction was as follows: 95°C for 3 min, followed by 45 cycles of denaturation at 95°C, annealing at 55°C, and extension at 72°C for 30 s each. A common threshold signal was chosen manually in the linear amplification range of all samples by inspecting the log-transformed fluorescence signals plotted against cycle number (Applied Biosystems, 7900HT Fast Real-Time PCR System). For each gene, the relative expression ratio was calculated using the ddCt method correcting for amplification efficiency of each primer pair and using *Gapdh* and *Hprt1* as reference genes.

In situ hybridization
E13.5, E15.5 and E17.5 C57BL/6 mouse brains were rapidly removed and quickly frozen with dry ice. Brains were cryosectioned (14 µm) and mounted onto Superfrost Plus slides (Thermo Fisher Scientific), air-dried, and stored desiccated at -80^{0}C. Non-radioactive *in situ* hybridization was carried out as described by Pasterkamp et al. (2007). Primers for *Nkx2.1* were forward primer: TCCACGCGCTTCTACTTTTT and reverse primer: TAAGCTTGGGAACCCATTTG. Primers to generate *Fzd3* and *Wnt5a in situ* probes were as described (Fenstermaker et al, 2010).

Immunohistochemistry
Cryosections (25 µm) were generated from *D2-EGFP* and *M4-EGFP* mice at E13.5, E15.5, E17.5 and P7 and stained immunohistochemically with the following primary antibodies: rabbit anti-EGFP (1:500, Molecular Probes, A11122), chicken anti-EGFP (1:500, Abcam AB13970), rabbit anti-DARPP32 (1:500, Santa Cruz SC-11365), rabbit anti-Nkx2.1 (1:2000,Biopat), or rabbit anti-TH (1:1000,Pelfreeze), mouse anti-LacZ (1:500, Promega). Alexa secondary anti-mouse, rabbit or chicken antibodies were used (1:500, Sigma). Slides were counterstained with fluorescent DAPI (Sigma). Slides were washed extensively and embedded in Mowiol (Sigma). Staining was visualized using a Zeiss Axioskop 2 microscope or by confocal laser-scanning microscopy

Dissociated neuron cultures
The striatum was microdissected from E15.5 *D2-EGFP* and *M4-EGFP* embryos. Tissue from several different embryos was pooled and incubated in trypsin for 20 min at 37°C. The tissue was then triturated with fire polished glass pasture pipets and trypsin activity was inhibited with 20% fetal calf serum. Then, cells were pelleted by mild centrifugation and re-suspended in Neurobasal Medium supplemented with B27, penicillin/streptomycin, and glutamine. A single cell suspension was generated by putting the cells through a 70 µm filter after which cells were plated on poly-D-lysine- and laminin-coated glass coverslips in a 12-well plate for 3 days. All cells were fixed with 4% paraformaldehyde for 10 min at room temperature and immunostained with the following primary antibodies: rabbit anti-Fzd3 (1:500, a gift from Jeremy Nathans) and chicken anti-EGFP (1:500, Abcam). Images were captured on a confocal laser microscope.

Neurite length quantification
The striatum was microdissected from E15.5 *Fzd3+/+; M4-EGFP* and *Fzd3-/-;M4-EGFP* embryos. Cells were dissociated and fixed as above. Cells were immunostained with anti-EGFP antibody and analyzed for changes in axon length. All cells were counterstained with DAPI. Embryos from one mother were pooled to obtain n=1. Three *Fzd3+/+; M4-EGFP* and *Fzd3-/-;M4-EGFP* animals were used and 100 neurons were analyzed per n. Neurons were imaged with a Zeiss Axioskop 2 microscope and analyzed with Zeiss Axiovision software. Axon length was measured from the cell soma to the tip of the neurite. Data were statistically analyzed by one-tailed student T-Test and represented as means ±SEM.

Results

Ontogeny of striatopallidal and striatonigral axon projections

SP and SN neurons are difficult to identify due to their complex organization in the striatum. Both neuronal types are intermingled and are either clustered together forming "patches" or dispersed around the patches in the "matrix" with no clear distinction between SP and SN neurons (Gerfen, 1992). To examine the trajectories of SN and SP axons we used BAC transgenic *D2-EGFP* and *M4-EGFP* mice. Using simple anti-EGFP immunostaining, we examined both trajectories at several developmental stages. SP axons were clearly visible at E13.5 and started to extend in the ventral direction within the striatum. At E15.5, a few SP axons had reached the GP but at E17.5 the GP was prominently innervated by SP axons (Fig. 1A, C, and E). For tracking SN projections, we used anti-tyrosine hydroxylase (TH) immunostaining to identify the substania nigra, the target of SN axons. At E13.5, TH-positive axons approached the striatum and SN axons had begun to exit the striatum. By E15.5, SN axons grew through the GP and were extending towards the ventral telencephalon. As SN axons exited the striatum they aligned with incoming TH-positive axons. At this stage, the first SN axons were innervating the substania nigra. By E17.5, the number of SN axons extending along TH+ axons and innervating the substania nigra had dramatically increased (Fig. 1F). At P7, both the GP and substantia nigra were heavily innervated by SP and SN axons (Fig. 1G, H)

Molecular profiling of Frizzled receptors in striatopallidal and striatonigral neurons

SP and SN axons form two distinct axon pathways but little is known about the axon guidance cues involved in the formation of these tracts. The Fzd family of Wnt receptors is involved in the development of several axonal tracts (Lyuksyutova et al, 2003, Wang et al, 2002, Wang et al 2006, Liu et al, 2008). To examine whether Fzds also play a role in the development of the SP or SN tracts, we screened the expression of all Fzds in SP and SN neurons. Pure populations of SP or SN neurons were obtained from *D2-EGFP* and *M4-EGFP* transgenic mice by FACS. RNA from these neurons was then used to quantify relative expression of D2, M4 and Fzd receptors at E15.5 and E17.5 (Fig. 2). Of the Fzds tested, only *Fzd2* and *Fzd3* were detected at significant levels and present in both neuronal subtypes. Both *Fzd2* and *Fzd3* have been implicated in axon guidance (Rodriguez et al, 2005, Wang et al, 2002). Interestingly, Fzd3 has been implicated in the guidance of other axonal tracts running along the anterior-posterior (A-P) axis (Lyuksyutova et al, 2003; Fenstermaker et al, 2010). Therefore we hypothesized that Fzd3 contributed to the guidance of SP and SN axon along the A-P axis.
Next, we examined *Fzd3* mRNA expression in E13.5, E15.5 and E17.5 coronal sections to find *Fzd3* expression within the striatum throughout development. At E13.5, *Fzd3* was more intensely expressed in the subventricular zone and at later stages equally expressed throughout the striatum (Data not shown). We further confirmed Fzd3 expression by immunostaining of tissue sections and dissociated D2-EGFP and

M4-EGFP neurons with anti-Fzd3 antibodies (Fig. 3). Fzd3 was expressed in M4-EGFP positive neurons in tissue sections but absent from M4-EGFP neurons lacking Fzd3 (Fig. 3A-I). In addition, Fzd3 was intensely expressed in dissociated D2 and M4 neurons and co-localized with EGFP fluorescence in the cell soma and all neurites (Fig. 3J-O).

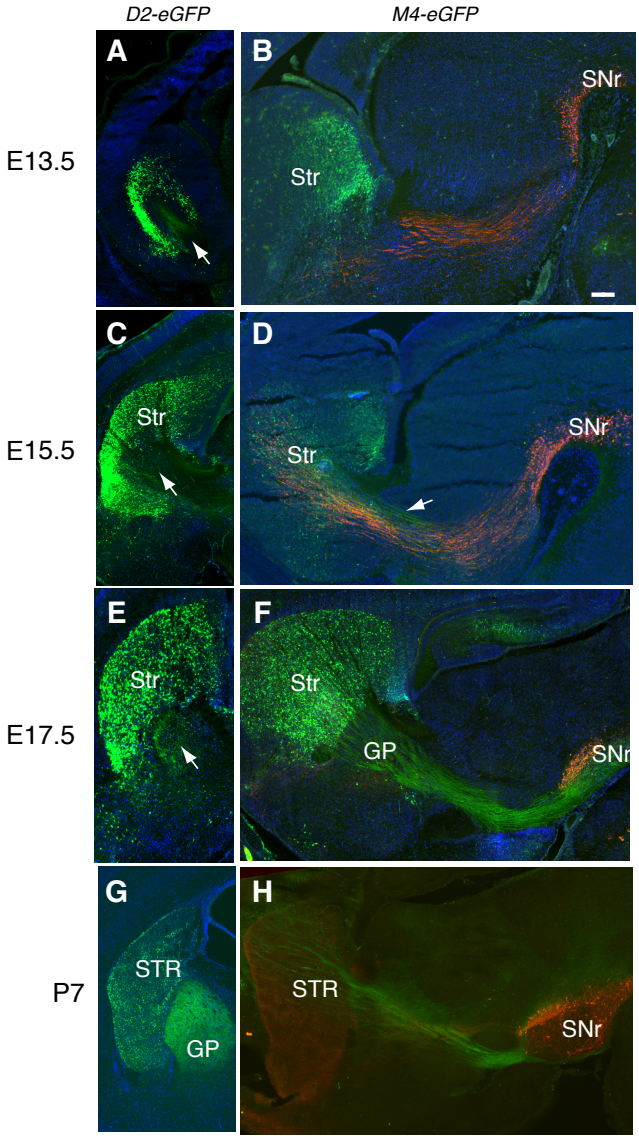

Figure 1. Development of striatopallidal and striatonigral axonal tracts
Immunohistochemistry for EGFP (green) and tyrosine hyrdroxylase (red) in sections from D2-EGFP (coronal; A, C, E, F) and M4-EGFP (sagittal; B, D, F, H) mice at E13.5, E15.5, E17.5 and P7. (A) At E13.5, SP neurons are present in the striatum and their axons have bundled together, projecting towards the ventral striatum (arrow). (B) At E13.5, SN neurons are present in the striatum with small protrusions directed towards the ventral striatum. At this stage, TH+ positive fibers approach the striatum. (C) At E15.5, the first SP reaches the GP (arrow). (D) By E15.5, SN axons are projecting to their midbrain target and are aligned with TH+ positive fibers (arrow). (E) By E17.5, many SP axons have reached the GP (arrow). (F) The growth of SN tract through GP is clear by E17.5. and many SN axons have reached the SNr. (G, H) At P7, the development of the SP and SN tracts is complete with dense innervation of the GP and SNr, respectively. (SP) striatopallidal tract, (SN) striatonigral tract, (SNr) substantia nigra pars reticulata, (STR) striatum, (GP) globus pallidus. Scale bar= 40 µm (A, C, E, G); 80µm (B, D, F,H).

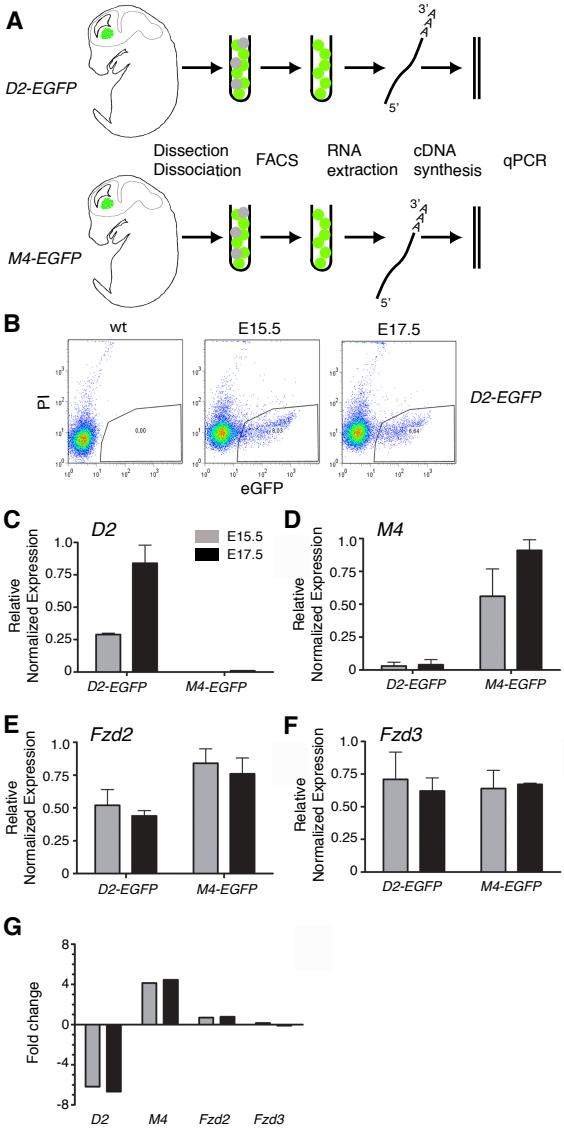

Figure 2. Fzd2 and Fzd3 are expressed in striatopallidal and striatonigral neurons
(A) Schematic showing the procedure to obtain cDNA from SP and SN neurons. Striata dissected from D2-EGFP and M4-EGFP transgenic mice were enzymatically dissociated and purified to EGFP populations using FACS. RNA was extracted and converted into cDNA for qPCR. (B) Scatter plot shows distribution of EGFP$^+$ and PI$^-$ MSNs of D2-EGFP at E15.5 and E17.5 in comparison with reference tissue (wt or Bl6). EGFP$^+$ MSNs are outlined in the bottom right quadrant (C, D, E, F) Relative transcript levels of D2, M4, Fzd2 and Fzd3 in D2-EGFP$^+$ and M4-EGFP$^+$ cells determined by qPCR at E15.5 and E17.5 (C) D2 was expressed in D2-EGFP$^+$ but not in M4-EGFP$^+$ cells. (D) M4 is expressed in M4-EGFP$^+$ but not D2-EGFP$^+$ cells. Expression levels of D2 and M4 had increased from E17.5 compared to E15.5. (E), (F) Fzd2 and Fzd3 are expressed in D2-EGFP$^+$ and M4-EGFP$^+$ neurons at E15.5 and E17.5. (G) Shows the fold change of D2, M4, Fzd2 and Fzd3 transcript levels in the E15.5 and E17.5 wt striatum. (D2) dopamine receptor D2, (M4) muscarinic acetylcholine receptor 4, (Fzd) Frizzled.

MSN axons fail to enter the globus pallidus in *Fzd3*-/- mice

Due to the prominent expression of Fzd3 in SP and SN neurons, we first studied the general effects of Fzd3 in MSNs. All MSNs express dopamine- and cyclic AMP-regulated phosphoprotein (DARPP32) (Shin et al, 2011). Examination of DARPP32 expression in *Fzd3* wt and *Fzd3*-/- brains showed that all MSN axons stalled in the caudal striatum in the absence of Fzd3 (Fig. 4A-I). We suspected that MSN axons stalled at the GP region and used *Nkx2.1* mRNA expression to localize the GP. Co-immunostaining with anti-DARPP32 and anti-neurofilament antibodies showed MSNs axons entering the GP in control mice. Neurofilament positive cortical and thalamocortical axons axons ran adjacent to the GP through the corridor region

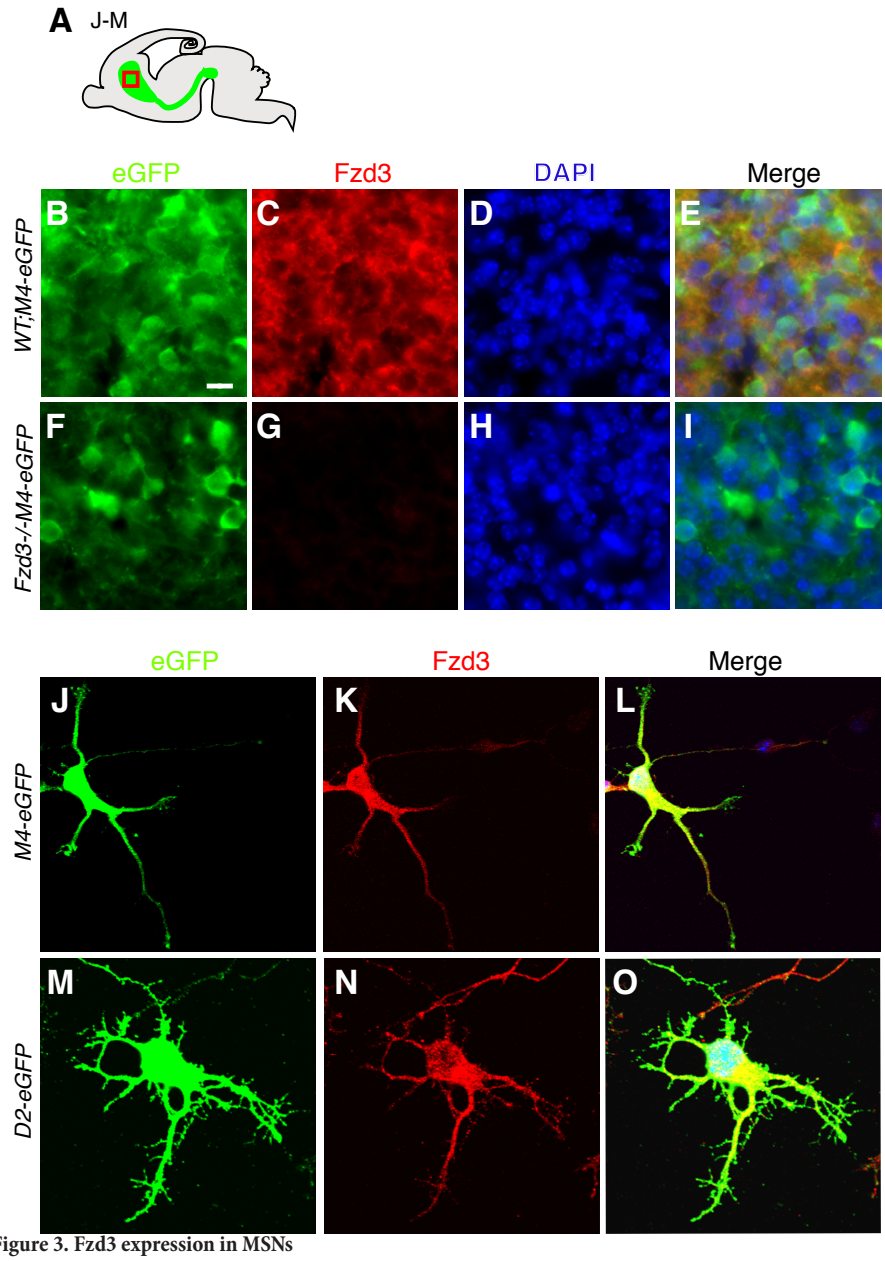

Figure 3. Fzd3 expression in MSNs

(A) Schematic showing the region used to obtain tissue sections or dissociated neurons. (B-I) Coronal sections of wt; M4-EGFP mice stained with anti-EGFP (B), anti-Fzd3 (C) and DAPI (D). (E) Merged image shows that Fzd3 is co-expressed in wt;M4-EGFP neurons. Coronal section of *Fzd3-/-; M4-EGFP* stained for anti-EGFP (F), anti-Fzd3 (G), and DAPI (H). (I) Merged image shows that Fzd3 protein is not expressed in *Fzd3-/-; M4-EGFP* neurons. (J-O) Dissociated M4-EGFP and D2-EGFP neurons stained with anti-EGFP and anti-Fzd3 antibodies. Merged image in (L) shows M4-EGFP neurons co-stained with anti-EGFP and anti-Fzd3 and (O) D2-EGFP neurons co-stained with anti-EGFP and anti-Fzd3. (D2) dopamine receptor D2, (M4) muscarinic acetylcholine receptor 4, (Fzd3) Frizzled3. Scale bar= 40 μm (B-I) and = 80 μm (J-O).

(López-Bendito et al, 2006) (Fig. 4J, K). In *Fzd3-/-*, few cortical and thalamocortical axons could be detected. In addition, MSN axons halted in the caudal striatum at the boundary of the GP, marked by *Nkx2.1* expression (Fig. 4L, M). In addition to axon guidance defects, we noted that the organization of the GP was changed in *Fzd3-/-* (Fig. 4K,M). In *Fzd3-/-*, the area containing *Nkx2.1* expression was smaller as compared to wt. In addition, Nkx2.1 positive cells were evenly distributed in the wt GP, but in the *Fzd3-/-* a *Nkx2.1* gradient was discernable with more prominent labeling of the medial as compared to the lateral GP (Fig. 4K, M).

Striatopallidal and striatonigral tracts fail to form in *Fzd3* mutant mice

To determine the role of Fzd3 specifically in SP and SN neurons and axon tracts, we generated *Fzd3-/-; D2-EGFP* and *Fzd3-/-; M4-EGFP* transgenic mouse lines. We examined *Fzd3-/-; D2-EGFP* and *Fzd3-/-; M4-EGFP* mice at E13.5, E15.5, and E17.5 (Fig. 5). For SP projections, defects were detected as early as E13.5. In the wt, SP axons start to fasciculate and grow towards the GP at E13.5. In *Fzd3-/-;D2-EGFP* mice, SP axons aberrantly accumulated near the GP (Fig. 5A-C). This phenotype persisted until E17.5; normally at this stage SP axons have entered the GP. However, in *Fzd3-/-; D2-EGFP* the SP axons did not enter the GP and accumulated at the caudal border of the GP (Fig. 5D, E). To examine the role of Fzd3 during SN pathway formation we examined the SN tract three levels, in the striatum, in the telencephalon en route to the substantia nigra, and in the midbrain at E17.5. In the striatum of *Fzd3-/-; M4-EGFP* mice, SN axons accumulated at the border of the striatum and the GP (data not shown). Furthermore, no SN tract was detectable in the telencephalon in the absence of Fzd3 (Fig. 5G, H, J, K). Finally, no SN axons were detected in the midbrain in *Fzd3-/-; M4-EGFP* mice (Fig. 5I, L).

Axonal outgrowth of striatonigral neurons is normal in *Fzd3-/-*

Since MSN axons halted at the GP in *Fzd3-/-* mice and fewer axons were observed, we wondered if MSN axon outgrowth was changed in *Fzd3-/-* mice. We cultured *Fzd3* wt and *Fzd3-/-* striatal neurons and immunostained these dissociated cultures for DARPP32 but found no significant difference in neurite length (Fig. 6A). We also examined neurite length in *Fzd3+/+; M4-EGFP* and *Fzd3-/-; M4-EGFP* but no differences in neurite length, branching or morphology were observed in *Fzd3+/+; M4-EGFP* as compared to *Fzd3-/-; M4-EGFP* mice (Fig. 6B).MSN axons innervate and pass through the GP in wt mice (Fig. 7A, B) but no MSN axons were detected in the GP of *Fzd3-/-* mice (Fig. 4L). Since Fzd3 and Celsr3 work together in assembling several forebrain axon tracts (Zhou et al, 2008), we also examined potential defects in MSN axon projections in *Celsr3-/-* mice. At E17.5, MSN axons, visualized with anti-DARPP32 antibodies, accumulated at the border of the striatum and GP and in a characteristic patch in the GP of *Celsr3-/-* (Fig. 7C). This accumulation of DARPP32 axons in the GP was not observed in *Fzd3-/-* mice. DARPP32 is expressed by a specific subset of cortical neurons (Fig. 4) and it is possible that the axons accumulating in the GP in *Celsr3-/-* mice were in fact cortical axons. Neurofilament staining to mark cortical axons in *Celsr3-/-* mice revealed that indeed cortical axons enter and accumulate in the GP in the absence of Celsr3 (Fig. 7D).

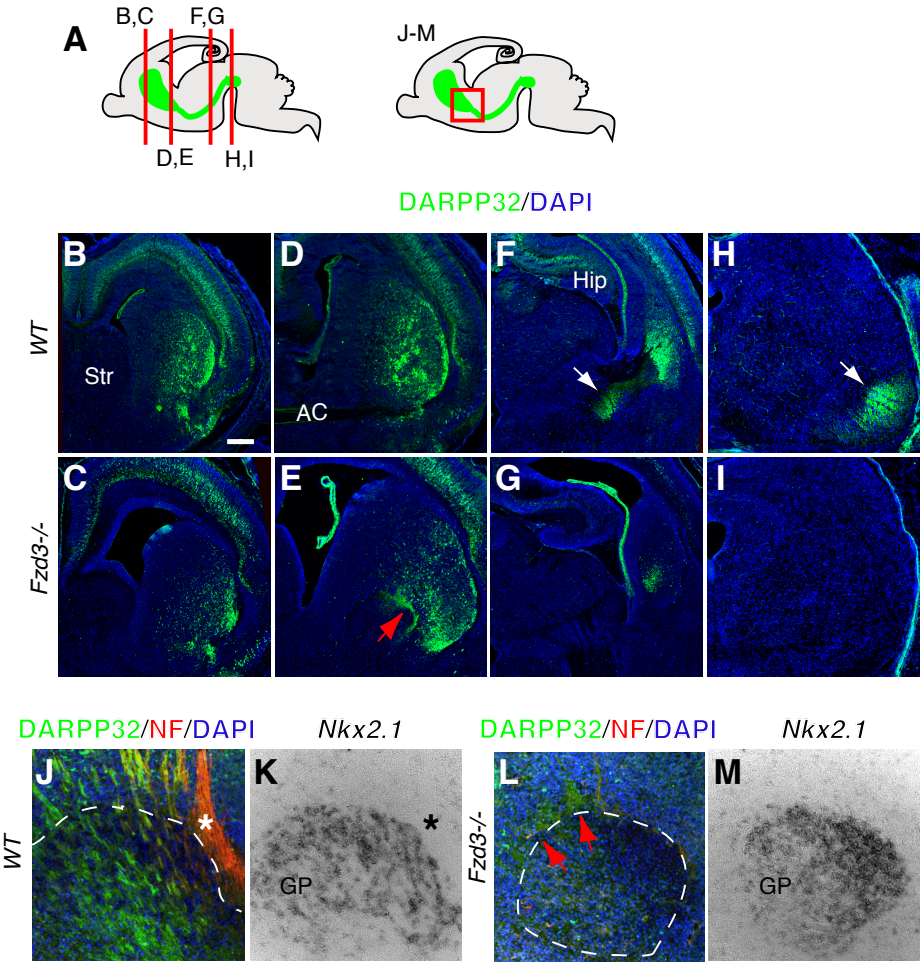

Figure 4. MSN axons fail to enter the GP in *Fzd3* mutant mice.
(A) Sagittal schematic of striatonigral system (green). Red lines indicate the location of the indicated panels. (B-I) Immunohistochemistry for DARPP32 (green) in E17.5 coronal sections. Sections are counterstained with Nissl in blue. In wt mice, MSN axons project through the GP (B, D) and the caudal telencephalon (F) to the substantia nigra (H). In contrast, in *Fzd3-/-* mice, axons stall at the GP (red arrow) and no striatonigral tract is formed in the telencephalon. (J, L) Immunohistochemistry for DARPP32 (green), neurofilament (NF, red) together with a Nissl staining in E17.5 coronal sections. In wt mice, MSN axons innervate the GP, while NF axons travel through the "corridor cell region" indicated by the *. In *Fzd3-/-* mice, MSN axons stall at the GP. While NF axons are almost absent. (K, M) *In situ* hybridization for *Nkx2.1*. The organization of the GP has changed in *Fzd3-/-* mice. Striatum (S), anterior commissure (AC), hippocampus (Hip). Scale bar= 80 µm (B-I) and 40 µm (J-M).

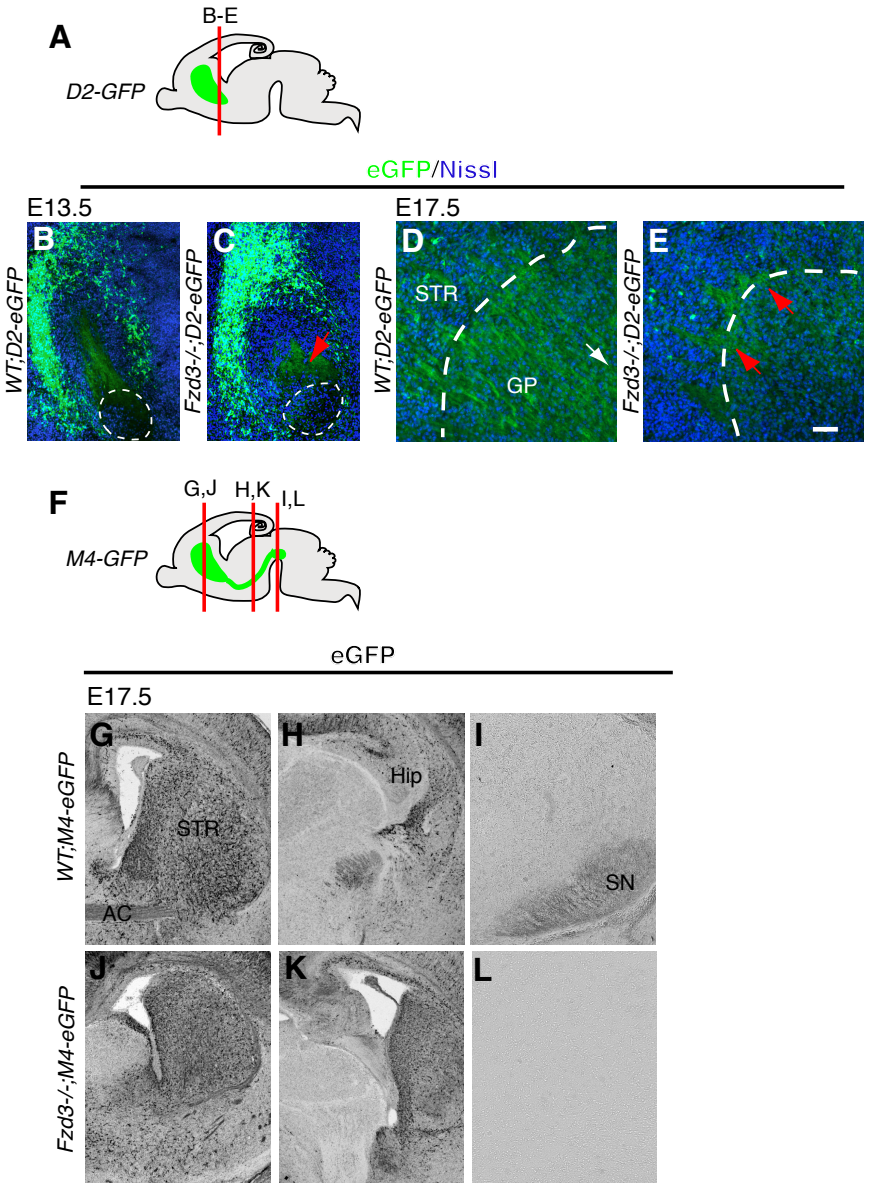

Figure 5. Striatopallidal and striatonigral tracts fail to form in *Fzd3* mutant mice.
(A) Sagittal schematic representation of the SP system (green). Red lines indicate the location of panels B-E. Immunofluorescence of EGFP (green) in coronal sections from E13.5 and E17.5 WT; *D2-EGFP* and *Fzd3-/-; D2-EGFP* mice. In *Fzd3-/-; D2-EGFP*, SP axons stall the edge of the GP and do not innervate the GP (red arrows in C and E). (F) Sagittal view of the SN system (green). Red lines indicate the location of panels G-L. (G-L) Immunohistochemistry for EGFP (DAB staining) on E17.5 coronal sections of WT; M4-EGFP and *Fzd3-/-; M4-EGFP* mice. The SN tract stalls at the GP in the absence of Fzd3. (D2) dopamine receptor D2, (M4) muscarinic acetylcholine receptor 4, and (Fzd3) Frizzled3, (DAB) Diaminobenzidine. Striatum (S), anterior commissure (AC), hippocampus (Hip). Scale bar= 80 μm (B-C) = 160μm (D-E) = 40μm (G-L).

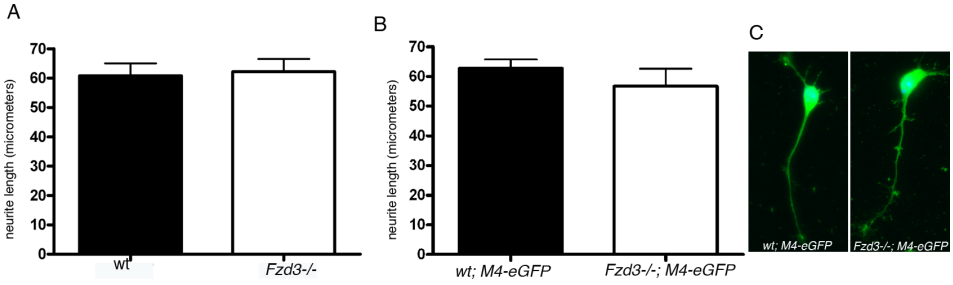

Figure 6. MSN axon outgrowth is normal in *Fzd3 mutant mice*
(A, B) Quantification of MSN neurite length in dissociated neuron cultures from wt and *Fzd3-/-* mice (A) and *wt; M4-EGFP and Fzd3-/-; M4-EGFP* mice (B). (C*)* EGFP fluorescence image of dissociated neurons from *wt; M4-EGFP* and *Fzd3-/-; M4-EGFP* mice. (M4) muscarinic acetylcholine receptor 4, (Fzd3) Frizzled3. Scale bar=80 μm.

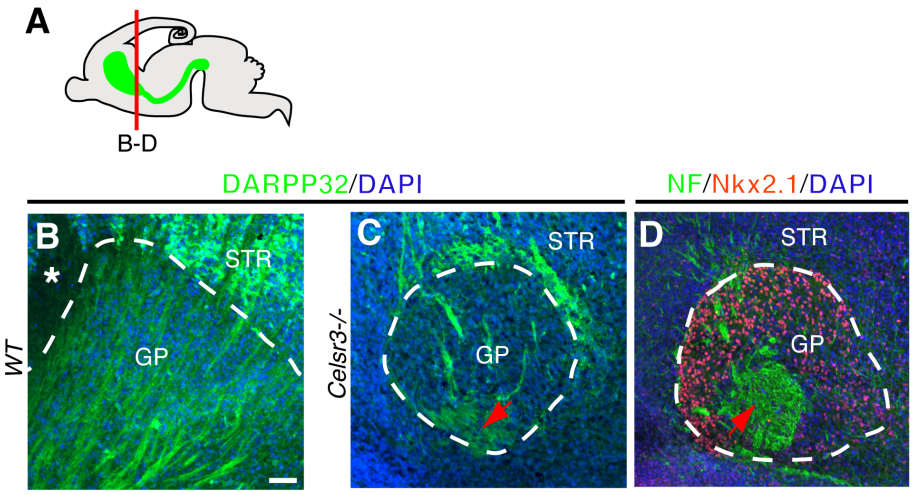

Figure 7. *Celsr3-/-* mice show defects in striatal and cortical axon projections
(A) Schematic representation of MSN axon projections (green). Red lines indicate the location of panels B-D. (B, C) Immunohistochemistry for DARPP32 (green) in E17.5 coronal sections of wt mice or *Celsr3-/-* mice. DARPP32 positive axons stall in the GP of *Celsr3-/-* mice (red arrow in C). (D) Immunohistochemistry for neurofilament (green) and Nkx2.1 (red) on a coronal section from a *Celsr3-/-* mouse at E17.5. Axons stalling in the GP are neurofilament positive cortical axons. Sections are counterstained with Nissl in blue. (NF) for neurofilament (STR) striatum, (GP) globus pallidus (Scale bar= 80μm)

Discussion

The generation of MSN neurons and their projections is a multi-step process. The molecular factors regulating the outgrowth, guidance and specification of MSN neurons into SP and SN neurons and axon pathways remain largely unknown. We characterized the development of the SP and SN pathways to determine critical decision points where SP and SN axons diverge into two distinct tracts. In addition, we demonstrate a

critical role for Fzd3 in the formation of both the SP and SN axon tracts. Our finding highlight that the GP is a permissive domain for SP and SN axons and that Fzd3 is required for MSN axons to enter the GP. It remains to be established why MSN axons stall at the GP in *Fzd3-/-* mice. Both the inability of MSN axons to detect permissive cues, such as Wnts, or changes in the organization of the GP in the absence of Fzd3 may prevent MSN axons from entering the GP.

Fzd3 is essential for the development of SN and SP axon tracts

Both SN and SP axons fail to enter the GP in absence of Fzd3. This shared phenotype demonstrates that Fzd3 is required for the proper development of both SP and SN axonal tracts. These results suggest SP and SN axons may use common molecular cues for their initial trajectory in the striatum. Fzd3 may affect MSN axon growth towards and into the GP in three different ways. First, MSN axons may use Fzd3 to detect permissive cues in the GP that allow them to enter this structure. Wnts are likely candidates for such a function. Fzd3 has been shown to work with Wnt4, Wnt5a and Wnt7b in axon guidance (Lyuksyutova et al, 2003, Fenstermaker et al, 2010). Our preliminary studies suggest that several Wnts are expressed in the GP, including Wnt5a (A.A.P. and R.J.P.).

Second, Fzd3 may affect the development of the GP, structurally or by regulating gene expression in GP cells. Such changes may render the GP less permissive for MSN axon growth. Molecular profiling of the GP shows that 50% of GP neurons are Nkx2.1-positive and the other 50% are Npas1-positive (Nobrega-Pereira et al, 2010). *Fzd3-/-* mice showed a disorganization of Nkx2.1-positive neurons characterized by a smaller GP and an altered distribution of GP cells. Perhaps these changes lead to another distribution of certain molecular cues ultimately leading to a failure of MSN axons to enter the GP. It is unknown how Fzd3 affects GP development. However, Fzd3 is expressed in the ventricular regions where GP cells originate and Fzd3 along with Celsr3 has been implicated in the migration of facial branchiomotor neurons (Vivancos et al, 2009, Wada et al, 2006, Pan et al, 2006). It is possible that GP cell migration is affected in *Fzd3-/-* mice.

A third possibility is that MSN axons require axon-axon interactions with other tracts for entering the GP. This model is discussed in the following section together with a hypothesis of how axon-axon interactions may assist MSN axons in reaching their distant targets in the midbrain.

Do MSN axons require mdDA axon projections?

The SN and SP axon pathways follow different trajectories and are functionally antagonistic (Lobo et al, 2006). Our results suggest that SP and SN axons share common cues for their initial outgrowth towards and into the GP. It remains unknown however which cues are required for subsequent pathfinding events, e.g. growth of SN axons from the GP to the midbrain. We observed that MSN and mdDA axons grow in close proximity suggesting that MSN axons may rely on mdDA projections for entering or exiting the GP or for extension through the telencephalon. It is therefore intriguing that previous studies have show that mdDA axons positively influence the growth of striatal axons and vice versa (Costantini et al, 1997, Snyder-Keller et al, 2008). It is

interesting to note that we have reported previously that mdDA axons do not grow towards the forebrain in *Fzd3*-/- mice (Fenstermaker et al, 2010). The inability of MSN axons to enter the GP or extend towards the midbrain may be a direct result of the lack of mdDA pathways. A similar model has been proposed for other interacting axon tracts, e.g. for developing thalamocortical and corticothalamic axons (i.e. the "handshake hypothesis") (Hirano, 2007). Further work is needed to examine whether MSN axons rely on mdDA axons or other axonal populations (e.g. serotonergic axons which are known to innervate the GP) for aspects of their pathfinding for example by genetically ablating mdDA neurons in mice or limiting Fzd3 deficiency to MSN neurons.

Fzd3 and Celsr3 work together during the formation of MSN axon tracts

The development of mdDA pathways requires a combination of different planar cell polarity receptors, i.e. Fzd3, Celsr3 and Vangl2 to detect Wnt gradients in the midbrain (Fenstermaker et al., 2010). By analogy, MSN axons stall at the GP in both *Fzd3*-/- and *Celsr3*-/- mice. This suggests that both receptors cooperate during the guidance of MSN axons. The involvement of Vangl2 remains to be determined. It should be noted however that the forebrain of *Vangl2* mutants displays an abnormal organization with a displaced striatum (A.A.P. and R.J.P., unpublished observations). Therefore, selective knockout of Vangl2 in the striatum is most likely required to address its role in MSN axon guidance. Although a large body of work indicates that Fzd3 and Celsr3 work together in the formation of forebrain axonal tracts (Zhou et al, 2008, Simons and Mlodzik, 2011), our data also hint at independent functions. In *Fzd3*-/-, mice cortical axons were found to stall at the GP together with MSN axons. In contrast, in *Celsr3*-/- mice cortical axons entered the GP and accumulated within this structure. Further studies are needed to establish the molecular and cellular basis of these differences in Fzd3 and Celsr3 function.

Conclusion

Overall our results show that SP and SN axons grow alongside to the GP and that during this early phase of axon guidance Fzd3 is required for GP entry. Further research is required to determine why MSN axons fail to enter the GP and which molecular cues are used for subsequent aspects of pathway formation. BAC transgenic mouse lines in which specific populations of MSN neurons are labeled will be a powerful future tool to identify such molecular signals.

Acknowledgements

We would like to thank Ger Arkestein for technical assistance during FACS, Alwin Derijck for help with qPCR and MMRRC for providing BAC transgenic mice. We are grateful to Rejji Kuruvilla and Jeremy Nathans for generously providing the *Fzd3* mutant mice and Jeremy Nathans for providing rabbit anti-Fzd3 antibody. This work was supported by The Netherlands Organization for Health Research and Development (ZonMW-VIDI and ZonMW-TOP), the International Parkinson Foundation,

the Human Frontier Science Program (Career Development Awards), and the European Union (mdDANeurodev, FP7/2007-2011, Grant 222999) (to R.J.P.). This study was performed in part within the framework of Dutch Top Institute Pharma Project T5-207 (to R.J.P.).

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Chapter 7

Discussion

To establish neuronal connectivity during development axons rely on molecular cues along the A-P and D-V axes of the brain. Dopaminergic (mdDA), striatal (MSN) and serotonergic (5HT) neurons form longitudinal axon tracts along the A-P axis of the CNS to reach their targets. All three tracts subserve important physiological functions and are being implicated in diseases such as depression and Parkinson's disease. Wnt proteins are secreted molecules that are expressed in gradients along the A-P axis of the developing nervous system inducing axon growth and guidance by binding axonal receptors such as Frizzled, and Ryk . The overall aim of this thesis was to examine the role of Wnts and their receptors in the A-P guidance of dopaminergic, striatal and serotonergic longitudinal axon tracts. The results reported in this thesis for the first time describe a role for Wnt/PCP signaling in the formation of mdDA, MSN and 5HT axon tracts. This chapter reviews these results, discusses the biological functions of Wnts and PCP receptors in the mdDA, MSN and 5HT systems, and briefly addresses possible implications for neural disease.

The globus pallidus is required for the development of striatopallidal and the striatonigral axon tracts.

GABAergic afferent projections of the striatum originate from medium spiny neurons (MSNs). MSNs make up approximately 95% of neurons in the striatum and form two distinct axon tracts, the striatopallidal (SP) tract and the striatonigral (SN) tract. The SP tract carries axons to the globus pallidus (GP), which then forms connections to the subthalamic nucleus (STN). STN axons then project to the substantia nigra pars reticulata (SNr). SN axons project directly from the striatum to the SNr. Thus, both tracts provide inputs into the SNr (Brazhnik et al, 2008; Gerfen, 1992). These pathways work together for motor control, where the direct pathway to the SNr promotes movement and the indirect pathway inhibits movement (Gerfen, 1992). MSN connectivity is also involved in motivation and reward seeking behaviors. Deregulation of MSN connectivity is implicated in Huntington's diseases, Parkinson's disease, drug addiction, depression and schizophrenia (Graybiel et al, 2008; Malenka et al, 2008; Baunez and Gubellini, 2010).

The molecular cues that control the formation of the SP and SN pathways are largely unknown. So far, Semaphorin 3E (Sema3E) and its receptor plexinD1 and Protocadherin 10 (Ol-pc) have been implicated in MSN pathway development. Sema3E and plexinD1 work together in the pathfinding of striatonigral axons. In the absence of Sema3E or plexinD1, striatonigral axons are misrouted (Chauvet et al, 2007). Ol-pc is essential for the outgrowth of striatal axons *in vitro* and *in vivo* (Uemura et al., 2007).

Because of this void of information on the mechanisms that control the development of striatal pathways, we set out to identify additional molecular factors that assist in striatal tract development in this thesis. In chapter 6, we demonstrated a critical role for Frizzled3 (Fzd3) in the formation of both the SP and SN axonal tracts. Analysis of *D2-EGFP* and *M4-EGFP* BAC transgenic mice crossed with *Fzd3* mutant mice, in which the SP and SN tracts are fluorescently labeled, indicated that absence of *Fzd3* results in the failure of both SP and SN axons to enter the GP. SP and SN axons stalled at the rostral and dorsal boundaries of the GP. In addition to axon guidance defects, we observed an abnormal organization of the GP in *Fzd3-/-* mice, where *Nkx2.1* expression was reduced as compared to wildtype (wt) mice. Furthermore, while *Nkx2.1*-positive cells are evenly distributed in

the wt GP, an Nkx2.1 expression gradient was found in *Fzd3-/-* mice with more prominent labeling of the medial as compared to the lateral GP. Based on these results two possible models can explain why MSNs axons fail to enter the GP in *Fzd3-/-* mice.
First, GP patterning may be changed in *Fzd3-/-* mice rendering the GP non-permissive for striatal axon growth. Second, attractive/permissive cues in the GP are no longer sensed by MSN axons in the absence of Fzd3 in striatal axons. Both models will be discussed in more detail in subsequent sections.

GP patterning

The GP is a final target for SP axons and an intermediate guidepost for SN axons. Therefore it is evident that the GP is crucial for the development of MSN circuitry. The GP is composed of cells that migrate from the lateral ganglionic eminence, medial ganglionic eminence and preoptic area (Bertran-Gonzalez et al, 2010). Canonical Fzd/Wnt signaling has been shown to regulate neurogenesis of the progenitors of Nkx2.1-positive neurons in the GP. The loss of β-catenin causes impaired growth and proliferation of the medial ganglionic eminence, which leads to a reduced number of Nkx2.1-positive cells (Gulacsi and Anderson, 2008). Furthermore, a gain-of-function mutation of β-catenin induces

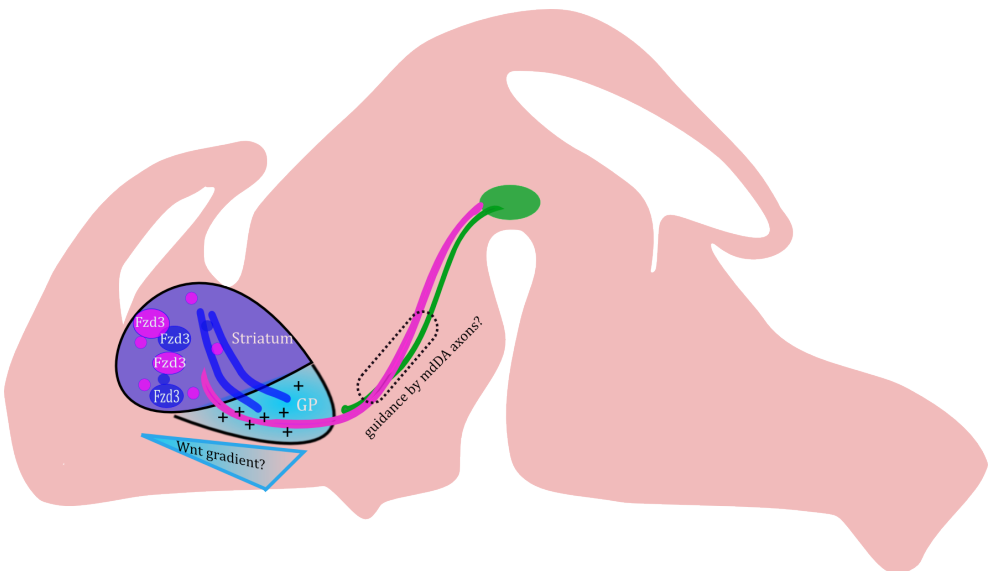

Figure. 1: SP and SN axons require Wnt/Fzd signaling to innervate the GP.
SP (blue) and SN (pink) neurons are intermingled in the striatum, either in clusters forming patches or distributed in the matrix. SP and SN axons initially grow towards the ventral telencephalon to innervate the GP. SP axons terminate in the GP and SN axons grow beyond the GP to synapse in the SNr (green). SP and SN neurons express Fzd3. An attractive gradient of Wnts may be present in the GP, which is sensed by Fzd3 expressed in SP and SN axons allowing them to enter the GP. Alternatively, the cellular organization of the GP may be crucial for MSN axon pathfinding. Postcrossing the GP, SN axons align with mdDA axons. We hypothesize that there is interdependency between SN and mdDA axons for their outgrowth in and out of the telencephalon, respectively. mdDA, mesodiencephalic dopamine, striatopallidal (SP), striatonigral (SN), the globus pallidus (GP), substantia nigra pars reticulata (SNr).

strong expression of *Nkx2.1* in the medial ganglionic eminence (Backman et al, 2005). These reports suggest that Fzd3/Wnt canonical signaling may be involved in the neurogenesis of GP progenitors, and therefore affect the organization of the GP. In *Fzd3-/-* mice, the size of the Nkx2.1-positive population appeared to be reduced. However, quantification of GP neurons in wt and *Fzd3-/-* mice is needed to establish whether there is a true difference in the number Nkx2.1-positive cells or merely a redistribution of these cells. Interestingly, Fzd3 has also been implicated in the migration of facial branchiomotor neurons (Vivancos et al, 2009; Wada et al, 2006; Pan et al, 2006). It is therefore possible that Fzd3 is involved in the migration of GP neurons. Defects in such migration may lead to a disorganization of the GP as observed in *Fzd3-/-* mice.
Findings of Uemura et al. (2007) suggest that the patterning of the GP is dependent on striatal axon growth (Uemura et al, 2007). Previous studies show that SP axons specifically project to the medial GP while SN axons project to the lateral GP (Kreitzer and Malenka, 2008; Durieux et al 2011). Therefore, the observed defects in GP organization in *Fzd3-/-* mice could be indirect, i.e. failure of MSN axons to innervate the GP may affect the regionalization of the GP resulting in the GP defects observed in *Fzd3-/-* mice. To establish a (in)direct role for Fzd3 in GP patterning, further experiments are needed to establish whether Fzd3 is expressed in the GP or the ganglionic eminence/preoptic area and then to selectively ablate Fzd3 from MSN neurons or the GP.

Axon guidance
The GP is a permissive domain for SP and SN axons. We hypothesize that Fzd3 expressed on SP and SN axons may function to sense attractive Wnts gradients in the GP enabling MSN axons to enter this structure. We base this hypothesis on the involvement of Fzd3 in axon guidance events in several other neuronal systems. For example, during the development of olfactory sensory pathways Fzd3 works together with Wnt5a (Rodriguez-Gil and Greer, 2008). Fzd3 is also expressed in spinal cord post-crossing commissural axons and guides them along the A-P axis by detecting an attractive Wnt4 gradient (Lyuksyutova et al., 2003). Our own findings demonstrate that detection of Wnt5a and Wnt7b gradients by Fzd3/PCP receptors is required for mdDA and 5HT axon guidance along the A-P axis (chapter 4). To examine whether Fzd3 is a receptor for Wnts on MSN axons, the presence of Wnts in the GP must be tested. Wnts previously described to act with Fzd3 are Wnt4, Wnt5a, Wnt7a and Wnt7b (chapter 4; Fenstermaker et al, 2010, Lyuksyutova et al., 2006; Rodriguez-Gil and Greer, 2008). Expression studies of all Wnts at E11.5 show that Wnt3a, Wnt4 and Wnt7b are expressed in the forebrain (Summerhurst et al, 2008). Wnts that are found to be expressed in the GP, can be tested in collagen assays with striatal explants to establish whether they can affect MSN axon guidance in a Fzd3-dependent manner as shown for mdDA axons in chapter 4.
After crossing the GP, SN axons require other axon guidance cues to grow towards and innervate the SNr. An important source of axon guidance cues for SN axons may be afferent mdDA axons. Previous studies have shown that mdDA axons positively influence the growth of striatal axons, and vice versa (Costantini et al, 1997, Snyder-Keller et al, 2008). Our findings in chapter 6 show that SN and mdDA axons grow in close proximity in the telencephalon. We hypothesize that SN axons require afferent mdDA axons to exit the GP and/or for extension through the telencephalon.

Overall our findings reveal that Fzd3 is required for the formation of SP and SN axon tracts. MSNs are progressively degenerating in Huntington's disease leading to impairments in motor and cognitive functions (Lee and Studer, 2010). The efficacy of cell replacement therapies for Huntington's disease is dependent on the ability of transplanted MSNs to form new functional circuitry (Kelly et al, 2009). Our findings suggest that Wnts are valuable molecular tools for guiding the axons of newly transplanted MSNs towards appropriate synaptic targets (e.g. the GP or SNr) in patients with Huntington's disease.

Wnts function as guidance cues during the development of mdDA neurons and their axonal projections

mdDA axons form mesolimbic, mesostriatal and mesocortical pathways. These neural circuits mediate motivational, motor and cognitive behaviors (Smidt and Burbach, 2007). Changes in mdDA connectivity are associated with various disorders such as Parkinson's disease, schizophrenia and drug addiction (Menke et al, 2011; Skidmore et al, 2011; Gu et al, 2008, Riddle and Pollock, 2003). Although different cues have been implicated in mdDA axon guidance (Van den Heuvel and Pasterkamp, 2008; chapter 2), the molecular control of several different aspects of mdDA pathway development remains to be established. In this thesis, we identify Wnts as guidance proteins for mdDA axons expressing Fzd3 and other PCP receptors (Fig. 2).

mdDA axon guidance

mdDA axons grow in the rostral direction to form axon projections in the forebrain. Nakamura et al. (2000) proposed that unidentified molecular mechanisms ensure the rostral growth of mdDA axons in the midbrain. Indeed, we discovered that Wnt/PCP signaling dictates the rostral orientation of mdDA axons at the level of the midbrain (chapter 3); The core PCP receptors Fzd3, Vangl2 and Celsr3 were expressed in mdDA neurons and axons. Additionally, Wnt5a and Wnt7b were expressed in opposite gradients in the midbrain. Using collagen assays, we demonstrated that Wnt5a is a chemorepellent and Wnt7b a chemoattractant for mdDA axons. Analysis of *Fzd3-/-*, *Vangl2-/-* and *Celsr3-/-* mice showed randomized mdDA axon growth and mdDA axons from these mice were unable to respond to Wnt5a and Wnt7b.

Interestingly, the phenotypes observed in the mdDA system of *Fzd3-/-, Celsr3-/-* and *Vangl2-/-* mice were similar but not the same (see chapter 4, Fig. 9). For example, when we examined *Fzd3-/-, Celsr3-/-* and *Vangl2-/-* mice at E12.5, fewer caudal projections were found in Celsr3-/- as compared to *Fzd3-/-* and *Vangl2-/-* mice. In contrast, at E17.5 defects in *Celsr3-/-* and *Fzd3-/-* mice were similar with many mdDA projecting caudally compared to the fewer caudal projections in *Vangl2-/-* mice. These differences may hint at compensation by other Celsr or Vangl family members at specific time points. It is for example possible that at early stages only Vangl2 is expressed, while at E17.5 both Vangl1 and Vangl2 are expressed. In such a model, Vangl1 could compensate for the absence of Vangl2 at E17.5 only. Alternatively, other molecular mechanisms may act to compensate for the loss of PCP receptors at specific developmental stages.

Despite marked caudal mdDA axon projections in *Fzd3-/-*, *Celsr3-/-* and *Vangl2-/-* mice, a subset of mdDA axons still projected rostrally, i.e. in their normal trajectory. This suggests that Wnt/PCP signaling is required for a subset of mdDA axons and that other subsets may be controlled by other cues. Indeed in 2009, two independent reports demonstrated a role for semaphorin 3F in the rostral growth of mdDA axons (Kolk et al, 2009; Yamauchi et al, 2009). In future studies it will be interesting to establish whether semaphorin 3F and Wnts affect different populations of mdDA axons and if the axons unaffected in PCP mutant mice are guided by semaphorin 3F. The rostrally growing axons in *Fzd3-/-* mice eventually stall at the ventral hypothalamus and fail to reach final targets such as the striatum and the medial prefrontal cortex (Bjorklund and Dunnet, 2007). This suggests that Fzd3 may also be required for mdDA axon guidance events in the forebrain.

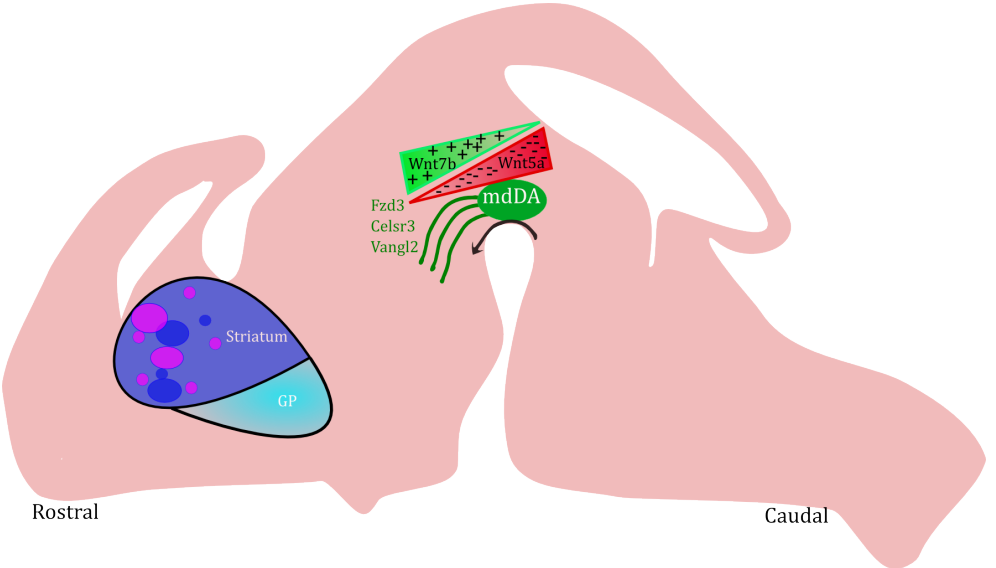

Figure 2: Wnts guide mdDA axons into the rostral direction.mdDA axons (green) grow in the rostral direction to form mesolimibic, mesostriatal and mesocortical pathways in the forebrain. The molecular mechanisms that ensure this rostral orientation were unknown (Nakamura et al, 2000). In this thesis, we describe that mdDA axons express core PCP receptors; Fzd3, Celsr3 and Vangl2 to sense Wnt gradients in the midbrain. Wnt5a is expressed in a high caudal, low rostral gradient and Wnt7b is expressed in a low caudal, high rostral gradient. mdDA axons are repelled by Wnt5a and attracted by Wnt7b. mdDA, mesodiencephalic dopamine; PCP, planar cell polarity.

Wnts as attractive and repulsive axon guidance cues

Wnts are able to induce both attraction and repulsion of monoaminergic axons. Wnt5a simultaneously repelled mdDA axons and attracted 5HT axons (chapter 4). This indicated that the biological response elicited by Wnts is dependent on the neuronal population. The patterns of Wnt receptor expression and intracellular machinery of these neuronal populations is unique and could contribute to the differences in their response to Wnt5a. The response of mdDA and 5HT axons may also be dependent on Wnt5a levels. Wnt5a is expressed in opposing gradients in the mdDA (low rostral-high caudal) and the 5HT (high rostral-low caudal) systems. Dose-dependent effects of Wnts have been described

for Wnt3, where differences in Wnt3 concentrations resulted in biphasic responses of retinal ganglion cells (Schmitt et al, 2006).
Within the mdDA system, Wnt5a and Wnt7b exerted opposite effects via Fzd3. How can the same axons be simultaneously attracted and repelled through the same receptor? It is possible that for unidirectional growth, axons undergo constant attraction and repulsion. *Wnt5a* and *Wnt7b* are expressed in opposite gradients in the mdDA system. Perhaps mdDA axons are repelled by high concentrations of Wnt5a in the caudal domain and gradually attracted by Wnt7b with increasing concentrations rostrally. Another possibility is that the Wnt5a gradient is generated first and repels mdDA axons away from the isthmus. At later stages, the Wnt7b gradient appears and attracts mdDA axons rostrally. A third explanation could be that in the complex environment of brain tissue mdDA axons in the caudal region are repelled by Wnt5a and switch off their responsiveness to Wnt5a as they extend rostrally now responding to Wnt7b only. Similar mechanisms have been shown for other neuronal systems. For example, commissural axons in the spinal cord are first attracted to the midline by netrins following which netrin responsiveness is turned off. Following midline crossing these axons respond to Slits but no longer to netrins (Kennedy et al, 1994; Sabatier et al, 2004).
Another issue that remains to be addressed is whether Wnt5a and Wnt7b use distinct receptor complexes to elicit repulsion and attraction, respectively. Although both Wnts require Fzd3 it is possible that other Wnt receptors such as Lrp5/6, Ryk and Ror are also involved (Charron and Tessier-Lavigne, 2005). In chapter 5, we show that Ryk can mediate Wnt5a chemorepulsive responses in mdDA axons *in vitro*. Previous work has shown that Ryk plays a prominent role in the axon guidance of cortical axons. Examination of *Ryk*-/- mice in these systems showed major axonal defects (Schmitt et al, 2006; Keeble et al, 2006; Li et al, 2009; Hutchins et al, 2011). However, *Ryk*-/- mice showed no defects in the mdDA system. This discrepancy between our *in vitro* and *in vivo* findings may be explained by compensation by other Wnt receptors such as Lrp5/6 or Ror proteins. *Lrp6* is expressed in the developing midbrain and *Lrp6*-/- mice show defects in mdDA neuron differentiation and midbrain morphogenesis (Castelo-Branco et al, 2010). The *Lrp6*-/- phenotype is similar to that found in *Wnt5a*-/- mice (Anderson et al, 2008). Ror2 is also a likely candidate to interact with Wnt5a in the midbrain. *Ror2* expression was found in the rat midbrain by RT-PCR and the non-neuronal phenotypes described in *Ror2*-/- and *Wnt5a*-/- outside are strikingly similar (Clayton and Sullivan, 2007; Oishi et al, 2003). Future examination of mdDA axons in *Lrp6*-/- or *Ror2*-/- mice or collagen gel assays with mdDA explants from *Lrp6*-/- or *Ror2*-/- mice co-cultured with Wnt5a producing cells will address whether Lrp6 or Ror2 are required for Wnt5a chemorepulsion.

Patterning of the mdDA neuron pool

We also showed that Fzd3 is required for the proper patterning of the mdDA system and that PCP signaling is most likely involved due to the fact that a similar phenotype was observed in *Celsr3*-/- mice (chapter 4). In *Fzd3*-/- and *Celsr3*-/- mice, the mdDA neuron pool expanded laterally due to an abnormal organization of the radial glia scaffold. It is unclear if the disorganization of the mdDA neuron pool is caused by a general expansion of the midbrain region or due to the malformation of the radial glia scaffold only. Although the patterning of the midbrain region is intact in *Fzd3*-/- mice, it is possible

that the midbrain has expanded along its mediolateral axis. Careful measurements of the mediolateral axis in wt and *Fzd3*-/- mice will be useful to clarify this point and further molecular studies are needed to identify how Fzd3 exerts a (in)direct effect on radial glia .

In all, our findings provide a new perspective on the role of Wnts in the mdDA system. As described for Huntington's disease in the previous section, in patients with Parkinson's disease, loss of mdDA neurons can be treated by transplanting new dopaminergic neurons (Cord et al, 2010). Wnts may help to guide newly formed axons to their appropriate synaptic targets. In addition, as Wnts and their receptors have been associated with various psychiatric disorders further knowledge of the functions and mechanisms of Wnts will also help to further understand and perhaps treat situations of perturbed connectivity that characterize these disorders.

Wnt/PCP signaling dictates 5HT axon orientation

This thesis for the first time examined the expression and function of Wnts in the 5HT system. 5HT ascending and descending axons form important connections that control motor function and cognitive behavior (Lucki, 1998). Deregulation of serotonergic connectivity is implicated in neural diseases such as depression, drug abuse and autism (Daubert and Condron, 2010; Dhonnchadha and Cunningham, 2008). Interestingly, the *Fzd3* gene has been associated with schizophrenia and mood disorders (Hashimoto et al, 2005, Yang et al, 2003, Zhang et al, 2004).
5HT ascending axons project to the midbrain and forebrain, while 5HT descending axons project to the spinal cord (Gaspar et al, 2003). We found that Wnt/PCP signaling determines the A-P guidance of both 5HT ascending and descending axons in response to Wnt gradients (Fig. 3). Genetic removal of PCP receptors resulted in an aberrant orientation of ascending and descending axons. Interestingly, our findings also suggest that the orientation of 5HT axons determines the direction of 5HT neuronal migration. This hypothesis is based on the manner by which 5HT neurons migrate in wt and *Fzd3*-/- mice. Neurons generally undergo radial and/or somal migration to reach their final destination. For radial migration, neurons align with radial glia as described for the mdDA system (chapter 4). Somal translocation is the movement of the cell body along the axon and is independent of radial glia. 5HT precursors migrate from the ventricular zone to the raphe nuclei by moving their cellular body along the axon. (Hawthorne et al, 2010). Time-lapse images show that 5HT neurons initially have a bipolar morphology with processes contacting the ventricular and pial regions. The cell body elongates and translocates from the ventricle to the pia. Axons form from the pial process. The cell body then translocates into the axon to reach its final target (Hawthorne et al, 2010).
Our results thus far rely on immunohistochemically stained sections at specific time points. To firmly establish that Wnt/PCP signaling regulates the orientation of 5HT axons, which consequently determines the direction of 5HT neuronal migration, time-lapse microscopy during the period of 5HT neuronal migration in PCP receptor mutant mice will be necessary. Notably, Pet-1, an ETS domain transcription factor, is an early marker for differentiated serotonergic neurons (Hendricks et al, 1999). For the time-lapse imaging studies suggested above, mice expressing enhanced yellow fluorescent protein

under control of the *Pet-1* promotor (ePet-EYFP) (Hawthorne et al, 2010) crossed with *Fzd3-/-*, *Celsr3-/-* or *Vangl2-/-* mice will be useful for visualization and quantification of defects in 5HT neuronal migration.

5HT axons were responsive to multiple Wnts (Wnt4, Wnt5a and Wnt7a) *in vitro*. In the hindbrain open-book assay, where the hindbrain is dissected and opened at the dorsal region to reveal the midline, beads soaked with Wnts were placed along the midline to test the axon guidance effects of Wnts on 5HT axons. In these open-book assays, Wnt4, Wnt5a and Wnt7a disturbed the rostral growth of 5HT axons. However, this assay does not reveal whether Wnts have attractive or repulsive effects on 5HT axons. In future experiments, the use of collagen assays of dissected rostral or caudal raphe explants co-cultured with selected Wnt proteins will clarify the nature of the observed effects. Such experiments will also reveal whether ascending and descending 5HT axons respond differently to Wnt proteins, as observed for mdDA axons.

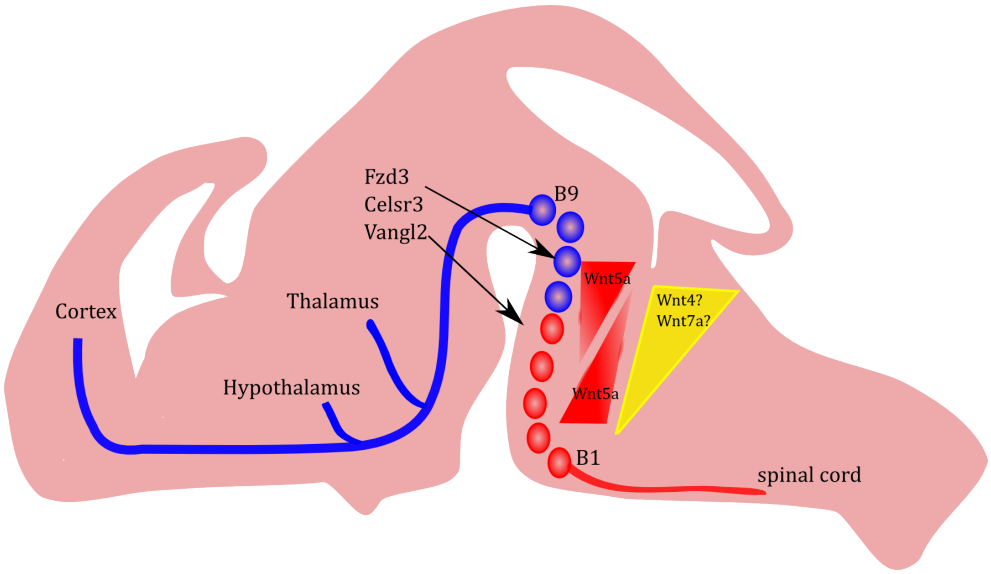

Figure 3: 5HT axons require PCP receptors to sense Wnt gradients for their proper orientation. 5HT ascending (blue) and descending (red) axons innervate the forebrain and spinal cord, respectively. Both axonal populations express Fzd3, Celsr3 and Vangl2. 5HT axons respond to Wnt4, Wnt5a and Wnt7a in vitro. Wnt5a is expressed in a gradient in the hindbrain. The expression patterns of Wnt4 and Wnt7a remain to the established. 5HT ascending and descending axons express Fzd3, Celsr3 and Vangl2 receptors to sense Wnt gradients to orientate them in the correct rostral or caudal direction.

Differences between ascending and descending 5HT projections

Axons of the 5HT system are commonly classified as ascending and descending based on the direction of their projections. These projections arise from nine sub-populations (B1-9) of 5HT neurons. 5HT neurons in B6-B9 form ascending projections to the midbrain and the forebrain. Neurons in B1–B5 form descending projections to the brainstem and spinal cord (Gaspar et al, 2003). Although all these neurons express the neurotransmitter serotonin (5HT), molecular and cellular differences exist between the different 5HT

nuclei. This is also evident from the defects observed in PCP mutant mice (chapter 4). The phenotypes of *Fzd3-/-, Celsr3-/-* and *Vangl2-/-* mice were different for ascending and descending axons. For example, in all PCP mutant mice the rostrally projecting ascending axons projected caudally and into rhombomere 4. However, descending axons showed striking differences in their phenotype. In *Fzd3-/-* mice, axons were shorter and projected randomly. Whereas in *Vangl2-/-* mice axons were highly fasciculated and in *Celsr3-/-* mice most descending axons projected normally. It is possible that these differences are the result of compensation by other PCP receptors in specific subsets of 5HT neurons, as discussed for mdDA neurons. For example, some 5HT populations may express Celsr1, Celsr2 and Celsr3 and while other neurons only express Celsr3. In this latter population, other Celsr proteins would not compensate genetic ablation of Celsr3. Since *Vangl2-/-* mice show a very unique phenotype of axonal hyper-fasciculation, not seen in other PCP mutant mice, we speculate that Vangl2 also acts independently of Fzd3 and Celsr3. Recently, Vangl2 has been discovered to form a receptor complex with Ror2 to sense Wnt5a gradients during the elongation of limbs (Gao et al, 2011). This exciting new finding supports the idea that Vangl2 can act independently.

Despite the dramatic axonal defects observed in PCP mutant mice, a subset of axons projected normally in *Fzd3-/-, Celsr3-/-* and *Vangl2-/-* mice. This is most likely due to the presence of other axon guidance cues. Slit1, Slit2, Growth-associated protein-43 (GAP-43) and protocadherin-alpha are involved in 5HT axon guidance and branching (Bagri et al, 2002; Donovan et al, 2002, Katori et al, 2009).

De-regulation of 5HT connectivity has been linked to a number of psychiatric conditions such as depression, anxiety and autism (Gaspar et al, 2003). Unfortunately, our current understanding of the mechanisms that underlie the development and maintenance of 5HT connectivity is rather rudimentary. Our findings on the 5HT system, showing that Wnts guide ascending and descending axon projections and therefore determine the direction of neuronal migration, not only help to understand how 5HT connections are established but also provide a framework for understanding how these pathways are changed in disease.

Final words

In this thesis we reported a novel role for Wnt proteins in the guidance of longitudinal axon tracts in the CNS. The observed axon guidance effects on mdDA, 5HT and MSN pathways required the interaction of Wnts with Fzd, Celsr3 and Vangl2 receptors. This implicates the PCP signaling pathway in the development of longitudinal axon tracts. Our findings highlight a marked redundancy of PCP and Wnt proteins during axon guidance whereby the function of individual Wnt and PCP proteins is compensated for by for example other Wnt or PCP proteins. Moreover, our data reveal the presence of several unidentified molecular mechanisms in Wnt signaling, e.g. the existence of different receptors to signal attraction and repulsion. Given the important physiological roles of the mdDA, 5HT and MSN circuits and their implication in psychiatric disease, our findings may eventually help to understand and treat situations of altered neuronal connectivity in these systems.

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Abbreviations

5HT	Serotonergic
A-P	Anterior-posterior
BAC	Bacterial Artifical Chromosome
Celsr	cadherin EGF LAG seven pass G-type receptor
D-V	Dorsal-ventral
D2	dopamine receptor D2
Fzd	Frizzled
GE	Ganglionic eminence
GP	Globus pallidus
mdDA	Mesodiencephalic dopaminergic
M4	muscarinic receptor M4
MSN	Medium spiny neurons
SN	Striatonigral
SP	Striatopallidal
STN	Subthalamic nucleus
SNr	Substantia nigra pars compacta
TH	Tyrosine hydroxylase
PCP	Planar cell polarity
Vangl	Vang-like
VTA	Ventral tegmental area

Nederlandse Samenvatting

Het menselijk brein bevat meer dan 10 biljoen neuronen die samen meer dan 10 triljoen verbindingen vormen. Voor de totstandkoming van deze verbindingen gedurende de ontwikkeling van het brein zijn zenuwceluitlopers (axonen) nodig die door de extracellulaire omgeving groeien om hun synaptische einddoel te bereiken, bijvoorbeeld een andere zenuwcel (neuron) of een spiercel. Gedurende dit uitgroeiproces wordt de richting van groei bepaald door zogenaamde axon sturende eiwitten in de extracellulaire omgeving. Longitudinale axonale verbindingen in de hersenen zoals de *strationigrale* (SN), *stratiopallidale* (SP), *dopaminerge* (mdDA) en *serotonerge* (5HT) zenuwbanen zijn immens complex en hebben een veelvoud aan axon sturende eiwitten nodig voor een succesvolle ontwikkeling. Schade aan deze banen wordt in verband gebracht met de ziekte van Huntington, de ziekte van Parkinson, drugs verslavingsproblematiek, depressie en schizofrenie. Er is echter verbazingwekkend weinig bekend over de axon sturende eiwtten die betrokken zijn bij de totstandkoming van de bovengenoemde banen. Wnt eiwitten zijn eiwitten die in het algemeen worden uitgescheiden in gradiënten langs de anterior-posterior (A-P) as van het zich ontwikkelende brein. Deze eiwitten induceren groei en sturing van axonen door zich te binden aan axonale receptoren zoals Frizzled en Ryk eiwitten. Dit proefschrift onderzoekt een nieuwe rol voor Wnt eiwitten en Wnt receptor eiwitten in de vorming van SP, SN, mdDA en 5HT zenuwbanen.

De SP en SN banen ontstaan van neuronen in het *striatum*. De SP baan bevat axonen naar de *globus pallidus* (GP), waar zich verbindingen vormen naar de *subthalamische kern* en vanuit daar verder met axonen die in verbinding staan met de *substantia nigra pars reticulata* (SNr). SN axonen groeien direct van het *striatum* naar de SNr. Onze resultaten laten zien dat SP en SN axonen langs de GP groeien en dat gedurende deze vroege fase van axon sturing de Frizzled3 (Fzd3) receptor nodig is voor toegang tot de GP. Onze resultaten laten voor de eerste keer zien dat SP en SN axonen gedurende specifieke gedeeltes van hun groei dezelfde eiwitten gebruiken.

mdDA axonen groeien in de rostrale richting (naar voren) om verbindingen te vormen in het voorste gedeelte van het brein, onder andere in het striatum en de cortex. We hebben ontdekt dat Wnt eiwitten deze rostrale groeirichting al bepalen ter hoogte van de middenhersenen. Door gebruik te maken van zogenaamde collageenkweken konden we aantonen dat Wnt5a een chemo-repellent eiwit (is afstotend) en Wnt7b een chemo-attractant eiwit (is aantrekkend) is voor mdDA axonen. De belangrijkste Wnt receptoren die betrokken zijn bij dit effect van Wnt5a en Wnt7b zijn Fzd3, Vangl2 en Celsr3. Deze eiwitten zijn aanwezig in dopaminerge axonen en noodzakelijk zijn voor het detecteren van Wnt eiwit gradienten.

Serotonerge (5HT) neuronen bevinden zich in de hersenstam en sturen axonen naar voren (anterior) naar de hersenen en naar achteren (posterior) naar het ruggenmerg. 5HT neuronen ontstaan in de ventriculaire zone van de hersenen en migreren naar hun uiteindelijke positie door dat het cellichaam van deze neuronen zich langs reeds gevormde uitlopers beweegt. Dit wordt somale translocatie genoemd. We hebben ontdekt dat de orientatie van het axon de uiteindelijke oriëntatie van het cellichaam van het 5HT neuron bepaalt. Met andere woorden axon sturing gaat somale trans-

locatie gedurende de ontwikkeling voor. Onze experimenten tonen aan dat de 5HT axonen gestuurd kunnen worden door Wnt4, Wnt5a en Wnt7a. Bovendien bevatten zij Wnt receptoren zoals Fzd3, Celsr3 en Vangl2 die nodig zijn om Wnts eiwitten te detecteren.

De bevindingen beschreven in dit proefschrift helpen niet alleen om te begrijpen hoe longitudinale zenuwbanen totstandkomen, maar geven ook inzicht in hoe deze banen veranderd zouden kunnen zijn in ziekten en hoe ze uiteindelijk gemanipuleerd kunnen worden ter behandeling van deze aandoeningen.

Curriculum Vitae

Asheeta Prasad was born in Lautoka, Fiji Islands on the 26th of September, 1980. She attended Jasper Williams Girls Primary School in Lautoka, Fiji Islands; followed by her high school education at Mount Roskill Grammar School in Auckland, New Zealand.
In 1999, she enrolled at the University of Western Sydney, Australia for a Bachelor of Biomedical Science degree. As part of her practical training, she worked at the Royal North Shore Hospital, Sydney, Australia at the Sutton arthritis research laboratory, supervised by Dr Chris Jackson and Dr Nathalie Buisson-Legendre . She was later employed there as a research assistant.
In 2004, Asheeta moved to back to New Zealand to work as a Medical Scientist at New Zealand human leukocyte antigen (HLA) typing laboratory.
In 2005, she started her Master's in Molecular Biology at University of Queensland, Brisbane, Australia which included research projects at Queensland Brain Institute on the Role of Slit2 in the developing Corpus Callosum, supervised by Dr Linda Richards, followed by a research project entitled, the Transcriptional Activity of p53 mutant commonly found in cancer at Manipal Life Sciences Centre, Manipal, India supervised by Dr K Satyamoorthy and Dr Ross Barnard.
After completing her Masters degree she worked at the National Cancer Institute, Amsterdam, in the research group of Dr Marten Lohuizen under the supervision of Dr Alexander Pietersen on the role of polycomb genes in stem cells. She joined the group of Dr Jeroen Pasterkamp in 2007 at the Utrecht Medical Centre, Utrecht, The Netherlands as a Phd student to study how Wnts guide longitudinal axon tracts in the brain. The results of her four year Phd are described in this thesis.

List of Publications

Prasad AA*, Fenstermaker A*, Bechara A, Adolfs Y, Tissir F, Goffinet A, Zou Y, and Pasterkamp RJ. Wnt-Planar Cell Polarity signaling controls the anterior-posterior organization of monoaminergic axons in the brainstem. **Journal of Neuroscience**, 2010 Nov 24;30(47):16053-64 .*shared first authorship,*Cover and featured article

Kolk SM, Gunput RA, Tran TS, van den Heuvel DM, Prasad AA, Hellemons AJ, Adolfs Y, Ginty DD, Kolodkin AL, Burbach JP, Smidt MP, Pasterkamp RJ.Semaphorin 3F is a bifunctional guidance cue for dopaminergic axons and controls their fasciculation, channeling, rostral growth, and intracortical targeting. **Journal of Neuroscience**. 2009 Oct7;29(40):12542-

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Prasad AA, Morello F, Adolfs Y, Pasterkamp RJ. Frizzled3 is required for the development of Medium Spiny Neurons. (Manuscript in preparation)

Acknowledgements

I started my phd with a 20kg backpack, a head full of dreams and a lonely planet guide of Europe. 4 years later I am taking back a lot of good friendships and memoirs.
To undertake a PhD in the Netherlands was a exciting challenge and turned out to be very rewarding.

The person who gets most of the credit is Dr Jeroen Pasterkamp, for taking me on board in the first place. Thank you for your kindness, support from presenting data to finalising this thesis. Guidance on the axonal tracts for motivation and reward. I was always impressed by the 24hour online help with instant feedbacks, the BBQ parties and skills in pouring a pint. I have learnt so much from you, I am forever grateful.

Professor Peter Burbach, thank you for providing insightful perspective on the results of this thesis, furthermore for your calm, positive and humble nature. It was wonderful to have you as my promoter.

Professor Marten Smidt for making academia seem fun. It was nice to have a joyous attitude around and of course your expert feedback on the discussions on the mdDA system was highly appreciated.

All the students I supervised, Alicia Nugent, Mariska Lier and Nathaly Espinoza, it is great to see you all continuing your careers in research.

To each and every member of the Pasterkamp group, Cathy, Natasha, Viviana, Koushik, Henk, Woutjan, Brendan, Geoffrey, Elisa, Maartje, Han, Mariken de Krom, Amila, Ella, Heidi, Oliver, Inge, Hugo, Eneda, Krista, Angela, Theresa, Anne, Ellen, Vicki and Ria for making a wonderful work environment.

In particular my paranimfen, Youri for keeping chivalry alive in the lab, cooking for me when I was sick. I can't say enough, you're a very kind person and my pillar at work.
Carlyn, there are too many highlights, varying form well executed lab pranks, bachelorette, wedding in NZ, the house parties, baby bonanza and the random "only one drink" call outs. You've been an amazing friend thought the years, pumping me up with confidence and a lot of booze.

My roomies, Yeping; only if RMI knew all the things you get up to, and Rahul, for all the entertainment! No day was mundane with you around.
Booooorel commitee Jules, Mytre and Marek for the eventful evenings including the fruitful vodka. Marek for also acting on the behalf of TNT, thank you for your movie reviews and an introduction course to Polish humour. Francesca, thank you for continuing the projects and being so caring. It was really nice to work with you.

Lars, during my first months at the department for showing me around the lab and ever since then teaching me Dutch vocabulary as well as showing me the local customs of the carnival.
Ruldof my amulet, I hope there are reindeer wings in heaven. Thanks for watching over my experiments.
Meneer van Halewijn, for your good morning greetings, making the 4 years seem like a European holiday. Your advice on the fine cuisine of the Brink (Krentebol met kroket, ossenworst en mustard dressing) was much appreciated. You tried to conceal your kindness, but I saw through. Thankyou!

Caroline (FFF) my secret keeper and partner in crime, thanks for a whole list of things. Mel aka CEO for the 7am squash matches, relaxing getaways etc, I always felt inspired after our chit-chats. Kim for showing me the alternative dutch lifestyle. Without good friends like you, I would have gone insane by now.
Annemarie aka baby whisperer for tips on raising a Dutch baby and your other expertise. Zeinab thanks for the Indesign tips and help with visa issues, a friend in need is a friend indeed.

My dutch family, the Blokker's and the De Jong's. Hartelijk bedankt voor de gezellige tijd. Mijn schoonmoeder, extremely awesome lady always with a smile and "no problem" motto.

Darling Desmond, my companion during the final phase of the thesis. You were there while I was writing my thesis, reading the manuscripts together. Every time I got sleepy you kept me awake and motivated. Seeing you grow and develop every day makes me more passionate about developmental biology.

Sjoey, you've been the foundation of the researcher I am today. When we met I was doubtful of pursuing an academic career, yet your encouragement, brave attitude towards life and belief in me were the key ingredients.

My mum, you've shown me how to live life to the most. You always made hard work seem easy, leaving me with the perception that everything is possible. Being at the other end of the world pushing the limits. There surely wasn't a day that I didn't miss you.
My dad and brothers; Bobby and Tony, for making me feel at home at whatever coordinates of the globe I was.
For the rest of the Whanau in Auckland and the Kavitis in Fiji, Bula Vinaka!