NEONATAL RESEARCH UNIT
DEPARTMENT OF WOMAN AND CHILD HEALTH
Karolinska Institutet, Stockholm, Sweden

WNT SIGNALING AFFECTS CELL ADHESION AND NEURONAL DIFFERENTIATION

Maria Lindqvist

Karolinska Institutet

Stockholm 2008

Published and Printed by Alfa Print AB
Cirkusgränd 2 172 63 Sundbyberg, Sweden

ISBN 978-91-7409-074-1

Till mina kottar Andreas, Axel och Anton
och till minnet av min morfar Lars Lindqvist

ABSTRACT

The mammalian central nervous system (CNS) consists of the brain and the spinal cord. The entire CNS is derived from the walls of a fluid-filled neural tube that is formed at an early stage of embryonic development. The tube itself becomes the adult ventricular system. Thus, by examining how this tube changes during the course of fetal development, we can understand how the brain is organized and how the different parts fit together.

The Wnts (Wingless-Int) seem to have important, but as yet not clearly understood roles as determinants of cell proliferation, migration and differentiation during both early and late phases of CNS development. This thesis investigates how Wnt signaling affects the cytoskeleton, cell adhesion and neuronal differentiation in the developing brain.

We begun by studying transgenic mouse embryos that overexpress Wnt7a in the neural stem cells. To our surprise the embryos displayed a disturbed neurulation. The neural tube shape and rigidity was affected. We demonstrated that this was due to reduced levels, and impaired distribution, of actin microfilaments, β-catenin, and N-cadherin at the neural tube adherens junctions. Formation of the neural tube is mainly accomplished by changes in cell shape and cell-cell interactions of committed ectodermal cells. We also showed that expression of the Wnt PCP signaling gene Vangl2 (Van gogh-like 2) was increased.

Wnt7a´s influence on neuronal differentiation was studied by investigation of β-tubulin III expression, a marker for early differentiated neurons. The pattern of β-tubulin III positive cells suggested a delay in neuronal development in Wnt7a overexpressing embryos.

Finally we investigated Vangl2's effect on the cytoskeleton. For this purpose we studied Vangl2 transfected HEK293T**,** MDCK and C17.2 cell lines, transgenic mouse embryos that overexpressed Vangl2, and loop-tail Vangl2 loss-of-function embryos. We were able to show that the components of the adherens junctions i.e. actin, β-catenin and N-cadherin, were affected in a similar way as in the Wnt7a overexpressing embryos. Investigation of signaling downstream of Vangl2 focused on the Rho GTPases, Rac1 and RhoA. Our results show that a balance between Rac1 and Vangl2 is essential for cell adhesion and mobility. Furthermore we could see that both a loss of function and a gain of function of Rac1/Vangl2 leads to changes in the actin cytoskeleton and disturbs adherens junctions.

Since Vangl2 and Wnt7a overexpressing mouse embryos displayed similar changes in the distribution of actin, N-cadherin, Scrb1 and β-catenin, we suggest in this thesis that Wnt7a can affect the cytoskeleton, cell adhesion and adherens junctions via Vangl2, Rac1 and P-JNK.

LIST OF PUBLICATIONS

I. Shariatmadari, M., Peyronnet, J., Papachristou, P., Horn, Z., Sousa, K., Arenas, E., and Ringstedt, T. (2005). Increased Wnt levels in the neural tube impair the function of adherens junctions during neurulation. Mol. Cell. Neurosci. *30*, 437-451.

II. Horn, Z., Papachristou, P., Shariatmadari, M., Peyronnet, J., Eriksson, B., and Ringstedt, T. (2007). Wnt7a overexpression delays beta-tubulin III expression in transgenic mouse embryos. Brain Res *1130*, 67-72.

III. Lindqvist, M., Horn, Z., Bryja, V., Papachristou, P., Ajima, R., Arenas, E., Yamaguchi, T P., Lagercrantz, H. and Ringstedt, T. (2008). Regulation of cell adhesion and adherens junctions by Vangl2. *Submitted manuscript.*

CONTENTS

LIST OF ABBREVIATIONS

BMP	Bone Morphogenetic Protein
BrdU	Bromodeoxyuridine
CNS	Central Nervous System
Dsh	Dishevelled
E	Embryonic day
FBS	Fetal Bovine Serum
Fz	Frizzled
GSK3	Glycogen Synthase Kinase 3
HEK	Human embryonic kidney
JNK	c-Jun NH2-terminal kinase
MDCK	Madin-Darby Canine Kidney
NF-AT	Nuclear factor of activated T-cells
PBS	Phosphate-buffered saline
PCP	Planar Cell Polarity
TCF/LEF	T-cell factor/lymphoid enhancing factor
TGF-β	Transforming growth factor-β
Vangl2	Van gogh-like 2
Wnt	Wingless-Int

1 INTRODUCTION

This thesis investigates how Wnt signaling affects the cytoskeleton, cell adhesion and neuronal differentiation in the developing brain.

1.1 DEVELOPMENT OF CENTRAL NERVOUS SYSTEM

Central Nervous system, CNS is a term that defines brain and the spinal cord. The entire CNS is derived from the walls of a fluid-filled neural tube that is formed at an early stage of embryonic development. The tube itself becomes the adult ventricular system. Thus, by examing how this tube changes during the course of fetal development, we can understand how the brain is organized and how the different parts fit together.

1.1.1 Formation of the Neural Tube

The embryo begins as a flat disk with three distinct layers of cells called endoderm, mesoderm, and ectoderm. The *endoderm* gives rise to the lining of many of the internal organs. From the *mesoderm* arise the bones of the skeleton and the muscles. The nervous system and the skin derives entirely from the *ectoderm.*

Our focus is on changes in the part of the *ectoderm* that gives rise to the nervous system: the *neural plate*. At this early stage, the brain consists only of a flat sheet of cells. The next event of interest is the formation of a groove in the neural plate, called the *neural groove*, that runs rostral to caudal. The walls of the groove, the *neural folds*, subsequently move together and fuse dorsally, forming the *neural tube.* The entire central nervous system develops from the walls of the neural tube. The process by which the neural plate becomes the neural tube is called *neurulation* (Smith JL, Schoenwolf GC. 1997). Thus, formation of the neural tube is mainly accomplished by changes in cell shape and cell-cell interactions of committed ectodermal cells, whereas cellular proliferation contributes up to this point only to a minor extent. The cells in the junctional region of the neural folds and later of the neural tube form the *neural crest cells,* which will migrate away and generate the peripheral nervous system, the pigment cells of the skin and several other cell types (Bonner-Fraser and Fraser, 1989; Fraser and Bronner-Fraser, 1991).

A common birth defect is the failure of appropriate closure of the neural tube. Failure of the anterior neural tube to close leads to anencephaly, while failure to close the caudal pore results in *spina bifida*. *Craniorachischisis* describes a condition where the neural tube is open from the hindbrain and caudally. The frequency of neural tube defects is affected both by chemical compounds such as the antiepileptic drug valporic acid and by genetic determinants , i.e. neural tube defects are more common in certain human populations.

1.1.2 Patterning in the early CNS

In mammals, it is clear that a first crude pattern along the rostro-caudal axis of the emerging neuroectoderm is already established during neural induction in the gastrula-stage embryo, although the precise molecular mechanism underlying it remain unclear. As the formation of the neural tube is completed, patterning is seen along the rostro-caudal axis as well as along the dorsal-ventral axis. (Lumsden & Krumlauf, 1996; Rubenstein & Beachy, 1998).

1.1.3 Neuronal Migration

The initial single cell layer of the neural tube is converted to a multilayer structure where the CNS stem/progenitor cells are confined to the inner ventricular layer and the newly formed daughter cells migrate outwards to specified layers of the developing cortical plate and spinal cord. Soon after the neural tube forms, many of the epithelial cells near its luminal surface begin to proliferate and give rise to neuroblasts. However a distinct group of neural cells, the *radial glial cells,* retain contacts with both the luminal and pial surface of the neural tube. In many regions of the developing brain the migration of neuroblasts and neurons is dependent on radial glial cells (Rakic 1972, 1974). The analysis of neuronal migration disorders occurring in humans and in animal models has provided a large amount of knowledge concerning migratory mechanism (Barkovich et al., 1992, 1996).

1.1.4 Genetic Control of CNS Development

Neurons are generated through transition from dorsal epithelium to neuroepithilial cells in the neural plate. Blocking bone morphogenetic protein (BMP) signaling appears to play a critical role in neural induction. (Hemmati-Brivanlou et al., 1994). Following neural induction, the developing CNS is regionalized along a rostral-caudal axis to establish the main regions: the forebrain, midbrain, hindbrain, and spinal cord (Lumsden and Krumlauf 1996). This segmented-type organization of the neural tube is achieved through the expression of a combination of soluble signals and transcription factors.

1.2 THE CYTOSKELETON

For cells to function properly, they must organize themselves in space and interact mechanically with their environment. They have to be correctly shaped, physically robust, and properly structured internally. Euaryotic cells have developed all these spatial and mechanical functions to a very high degree, and they depend on a remarkable system of filaments called the *cytoskeleton*. Most animal cells have 3 types of cytoskeletal filaments that are responsible for the cells spatial organization and mechanical properties. *Intermediate filaments* provide mechanical strength. *Microtubules* determine the positions of membrane-enclosed organelles and direct intracellular transport. *Actin filaments* determine the shape of the cell's surface and are necessary for whole-cell locomotion. *Actin filaments* are 2-stranded helical polymers of the protein actin. The actin subunits assemble head-to-tail to generate filaments with a

distinct structural polarity. Actin filaments in animal cells are organized into 2 types of arrays: bundles and weblike (gel-like) networks. Although actin filaments are dispersed throughout the cell, they are most highly concentrated in the *cortex,* just beneath the plasma membrane. Cell crawling is a highly integrated process, dependent on the actin-rich cortex beneath the plasma membrane. Three distinct activities are involved: *protusion* , in which actin-rich structures are pushed out at the front of the cell; *attachment*, in which the actin cytoskeleton connects across the plasma membrane to the substratum; and *traction* in which the bulk of the trailing cytoplasm is drawn forward. For the actin cytoskeleton, diverse cell-surface receptors trigger global structural rearrangements in response to external signals. But all of these signals seem to converge inside the cell on a group of closely related monomeric GTPases that are member of *Rho* protein family; *Cdc42, Rac, and Rho.* The same Rho family proteins are also involved in the establishment of many kinds of cell polarity. Rho proteins act as molecular switches to control cell processes by cycling between an active, GTP-bound state and an inactive state. Activation of Rac promotes actin polymerization at the cell periphery leading to the formation of sheet-like lamelliopedia extensions and membrane ruffles which are actin-rich protrusions on the cell's dorsal surface. (Wong and Adler, 1993; Fanto et al., 2000; Schambony and Wedlich, 2007).

1.2.1 Cell Adhesion and Cell Junctions

Within the epithelium, the cells are attached to each other directly by cell-cell adhesions, where cytoskeletal filaments are anchored, transmitting stresses across the interior of each cell, from adhesion site to adhesion site. Cell-cell adhesions transmit stresses and are tethered to cytoskeletal filaments inside the cell. The structures of cell-cell adhesions are most clearly seen in mature epithelia. Anchoring junctions link cell to cell, typically via transmembrane cadherin proteins. Adherence junctions are anchorage sites for actin filaments. The central role is played by transmembrane adhesion proteins that span the membrane, with one end linking to the cytoskeleton inside the cell and the other end linking to other structures outside it. Proteins of the cadherin superfamily chiefly mediate attachment of cell to cell. The first three cadherins that were discovered were named according to the main tissues in which they were found. *E-cadherin* is present on many types of epithelial cells; *N-cadherin* on nerve, muscle and lens cells; and *P-cadherin* on cells in placenta and epidermis. (Ybot-Gonzales and Copp, 1999). Anchoring junctions between cells are usually symmetrical: if the linkage is to actin, for example, in the cell on one side of the junction, it will be to actin in the cell on the other side also. In fact, the binding between cadherins is generally homophilic (like-to-like). Unlike receptors for soluble signal molecules, which bind their specific ligand with high affinity, cadherins (and most other cell-cell adhesion proteins) typically bind to their partners with low affinity. Cadherins mediate highly selective recognition, enabling cells of similar type to stick together and to stay segregated from other types of cells.

1.2.2 Cadherins Control the Selective Assortment of Cells

The appearance and disappearance of specific cadherins correlates with steps in embryonic development where cells regroup and change their contacts to create new

tissue structures. As the neural tube forms and pinches off from the overlaying ectoderm, neural tube cells lose E-cadherin and acquire other cadherins, including N-cadherin, while the cells in the overlaying ectoderm continue to express E-cadherin.

1.2.3 Regulation of Epithelial-Mesenchymal Transition

The assembly of cells into an epithelium is a reversible process. By switching on the expression of adhesion molecules, dispersed unattached cells, often called *mesenchymal cells,* can come together to form an epithelium. Conversely, epithelial cells can change their character, disassemble, and migrate away from their parent epithelium as separate individuals. Such *epithelial-mesenchymal transition*s play an important part in normal embryonic development; the origin of neural crest is one example. Epithelial-mesenchymal transitions also occur as pathological events during adult life, in cancer.

1.2.4 Catenins Link Cadherins to the Actin Cytoskeleton

The extracellular domains of cadherins mediate homophilic binding. The linkage to the cytoskeleton is indirect and depends on a cluster of accessory *intracellular anchor proteins* that assemble on the tail of the cadherin. This linkage, connecting the cadherin family member to actin or intermediate filaments, include several different components. These components vary somewhat according to the type of anchorage but in general a central part is played by β-catenin and/or its close relative γ-catenin. At adherence junctions, a remote relative of this pair of proteins, *p120-catenin*, is also present and helps to regulate assembly of the whole complex. When p120-catenin is artificially depleted, cadherin proteins are rapidly degraded, and cell-cell adhesion is lost.

1.2.5 Adherens Junctions Coordinate the Actin-Based Motility of Adjacent Cells

Adherens junctions are an essential part of the machinery for modelling the shapes of multicellular structures in the animal body. By indirectly linking the actin filaments in one cell to those in its neighbors, they enable the cells in the tissue to use their actin cytoskeleton in a coordinated way. The prototypical example of adherens junctions occur in epithelia, where they often form a continous adhesion belt close beneath the apical face of the epithelium, encircling each of the interacting cells in the sheet. The actin bundles are linked via the cadherins and anchor proteins into an extensive transcellular network. This network can contract with the help of myosin motor proteins, providing the motile force for a fundamental process in animal morphogenesis- the folding of epithelial cell sheets into tubes, vesicles and other related structures.

1.2.6 Scaffold Proteins Organize Junctional Complexes

Many different Ig-superfamily members, cadherins, and other cell-cell adhesion molecules guide the formation of nerve connections and hold neuronal membranes

together at synapses. In these complicated structures, as well as at other cell types of cell-cell junctions, intracellular scaffold proteins containing multiple PDZ protein binding domains have an important role in holding the many different adhesive and signaling molecules in their proper arrangement (Hildebrand and Soriano, 1999).

1.2.7 A separate Signaling System Controls Planar Cell Polarity

Apico-basal polarity is a universal feature of epithelia, but the cells of some epithelia show an additional polarity at right angles to this axis: it is as if they had an arrow written on them, pointing in a specific direction in the plane of the epithelium. This type of polarity is called *Planar cell polarity.* Planar cell polarity is a part of non-canonical Wnt signaling.

1.3 THE WNT FAMILY

The Wnt genes encode secreted glycoproteins, usually 350-400 amino acids in length, that function in intercellular signaling. The degree of sequence identity in Wnt proteins is minimally 18%, including a conserved pattern of 23-24 cysteine residues (Cardigan and Nusse, 1997). To date the Wnt gene family comprise Wnt1 to Wnt19, although some family members are found only in certain species. In addition, many of the Wnt genes appear in closely related forms (like Wnt7a and Wnt7b).

The first insigths into the biological function and signaling mechanism of Wnt genes came from studies of their orthologue in fruit flies, Wingless. This has early morphogenetic functions (Nusslein-Volhard and Wieschaus 1980). Studies of nockout mice reveal that this is true also of the Wnt genes. For example, Wnt1-/- mice do not develop a midbrain, Wnt4a -/-mice lack kidneys and mice lacking Wnt7a have ventralized limbs.

During neural development, Wnts have diverse roles in governing cell fate (Dorsky et al., 1998; Gunnhaga et al., 2003; Nordstrom et al.,2002; Wilson et al., 2001) proliferation (Chenn and Walsh, 2002; Dickinson et al., 1994; Megason and McMahon, 2002 Willert et al.,2003) morphogenesis of the neural tube (Deardorff et al., 1998; Hume and Dodd, 1993), neuronal differentiation (Castelo-Branco et al., 2003; Hall et all., 2000; Hirabayashi et al., 2004; Krylova et al., 2002; Lee et al., 2004) migration, polarity, and death. In adults Wnts function in homeostasis, and inappropiate activation of the Wnt pathway is implicated in a variety of cancers.

There is also diverse group of secreted Wnt inhibitors. This include the so called frizzled like proteins (Frzb), Wnt-inhibitory factor-1 (WIF-1), Cerberus and Dickkopf.

For a long time, a large gap in the understanding of the mechanism of Wnt signaling was the lack of known Wnt receptors. In 1996 frizzled proteins were identified as Wnt receptors (Bhanot-Nathans, John Hopkins).

1.3.1 Wnt Signaling Pathways

The first identified Wnt signaling pathway, the Wnt/β-catenin, also designated as the canonical pathway (Cadigan and Nusse, 1997; Huelsken and Birchmeier, 2001; Patapoutian and Reichert, 2000), has been studied in much detail and is highly conserved among species. The binding of Wnt molecules to members of the frizzled family of cell-surface receptors activates the cytoplasmic protein dishevelled which in turn destabilizes the Axin-APC-GSK3 complex and prevents GSK3 phosphorylation of β-catenin. The amount of β-catenin in the cytoplasmatic pool, as well as its post-translational modification by phosphorylation, are important factors in the transduction of the Wnt signal. Stabilized β-catenin enters the cell nucleus and associates with LEF/TCF transcription factors, thereby initiating transcription of Wnt-target genes.

The non-canonical Wnt-signaling pathways consists of Wnt/Ca^{2+}, Wnt/JNK (PCP) (Veeman et al., 2003a,b). The Wnt/ Ca^{2+} pathway involves an increase in intracellular calcium release and activation of protein kinase C and CamKII. Elevated Ca^{2+} activates the phosphatase calcineurin, which leads to the dephosphorylation of the transcription factor NF-AT and its accumulation in the nucleus (Saneyoshi T et al., 2002). In the planar cell polarity pathway, frizzled activates JNK and directs asymmetric cytoskeletal organization and coordinated polarization of cells within the plane of epithelial sheets. Vangl2 (the mouse orthologue of Drosophila Strabismus) and Scrb1 are newly identified molecules that belong to the PCP signaling pathway (Montcouquiol *et* al., 2003; Lu *et* al., 2004; Montcouquiol *et* al., 2006). The PCP pathway has been shown to to control convergent extension movements. (Heisenberg et al., 2000; Wallingford et al., 2000).

Wnt signaling also interacts with other cell-cell signaling pathways. For instance does notch and wingless signalls collide. In drosophila the intracellular domain of notch has been shown to bind to the PDZ domain of dishevelled (dsh). Dsh interacts antagonistically with notch and therefore it has been suggested that dsh blocks notch signaling directly through binding of the intracellular domain of notch.

The TGF-β and Wnt pathways also cross talk in controlling certain developmental events. They can independently or cooperatively regulate LEF1/TCF target genes. DNA binding sites in the Xenopus twin promotor are required for synergistic activation by TGF-β and Wnt pathways (Letamendia A et al 2001).

The large size of the Wnt and Fz families, the fact that they signal through at least 3 different pathways and interact with several others, makes it plausible that they are responsible for a large spectrum of developmental events.

2 METHODS

A brief description of the methods and techniques used in the thesis is given below. For further details, see references, and Material and Methods in papers I-III.

2.1 GENERATION OF TRANSGENIC MICE

Nestin, an intermediary filament, is considered a marker of pluripotent neuronal stem cells. Results from several research groups, including our own, verify that constructs with the nestin gene results in a strong expression of the target gene between at least embryonic day 7,75 (E7,75) and E16,5. Expression continues in areas that develop later, such as the cerebellum. A large portion of the cells in the brain will be affected, since nestin is expressed in the ventricular zone, where most of them originate. Nestin positive cells can also be found in other areas, such as the cortical plate, since also glial precursor cells are nestin positive.

Tissue specificity of nestin expression is governed by enhancer elements located in the genes first and second intron. The promoter is not relevant. The first intron directs expression to developing muscle tissue, the second to neuronal stem cells. In the study I-III we use a vector (nes1852tk/lacZ) that contains only the second nestin intron, and has a documented function in directing expression to neuronal stem cells (Lothian et al 1997).

2.1.1 Generation of the Wnt-7a Construct

These steps were followed:

1) PCR on mouse E13 cDNA with primers spanning the entire length of the Wnt7a open reading frame. The primers used were fitted with restriction sites appropriate for cloning in the nes1852tk/lacZ vector.
2) Cloning of the resulting PCR product in a PBS SK- vector (with the chosen restriction sites).
3) Sequencing of the cloned fragment.
4) Subcloning of the Wnt7a fragment into the nes1852tk/lacZ vector.
5) The resulting product (nesWnt7a) were magnified, stripped of its plasmid component, and delivered to the Karolinska Institute Transgene Core Facility (MouseCamp) for injection into fertilized mouse oocytes.

2.1.2 Generation of the Vangl2 Construct

For this study a 1566 bp fragment spanning the open reading frame of Vangl2 and flanked by XhoI and HindIII sites was generated by PCR from a cDNA clone containing the Vangl2 coding sequence (I.M.A.G.E. Consortium [LLNL] cDNA CloneID 6509008;(Lennon et al., 1996)purchased from RZPD (http://www.rzpd.de/; RZPD CloneID IMAGp998J1714075Q3). The upstream primer was 5- TAA CTC GAG *ATG* GAC ACC GAG TCC CAG TA and the downstream primer was 5-GAC

AAG CTT TCA CAC AGA GGT CTC CGA (the XhoI respectively HindIII sites are underlined and the start codon is indicated in italics). The Vangl2 fragment was subcloned into pBluescript SK- (Stratagene, La Jolla, CA) and sequenced. It was then cut out and subcloned into the NotI site of the nes1852tk/lacZ vector. The resulting product (nesVangl2) were magnified, stripped of its plasmid component, and delivered to the Karolinska Institute Transgene Core Facility (MouseCamp) for injection into fertilized mouse oocytes.

2.1.3 Collection of Embryos

Pregnant female mice at defined stages post coitum (the morning of plugging was counted as E0.5), were sacrificed by servical dislocation and the embryos were fixed Embryos were fixed 2–4 h in 4% paraformaldehyde and cryoprotected overnight in 10% sucrose in phosphate-buffered saline (PBS), pH 7.4. The embryos were then embedded in mounting medium (Tissue-Tek, Ted Pella Inc, Redding, CA), rapidly frozen, and 10 μM sections were cut on a cryostat.

2.2 CELL CULTURE

The following cell lines were used: Human embryonic kidney (HEK) 293T, Madin-Darby Canine Kidney (MDCK) and C17.2 which is a neural stem cell generated from rat P4 . HEK 293T cells were cultured in Dulbecco's modified Eagle medium (DMEM; GIBCO, cat no. 11971) supplemented with 10% fetal bovine serum (FBS) and anitibiotics. MDCK cells in MEM (GIBCO, cat no. 21090) supplemented with 10% FBS, 2 mM L-glutamine, 1% non-essential amino acids, and antibiotics; and C17.2 cells in DMEM (GIBCO, cat no. 41966) supplemented with 10% FBS, 5% horse serum, 2 mM L-glutamine and antibiotics (all supplements from GIBCO).

2.2.1 Generation of the Vangl2-HA Construct

The Vangl2 fragment was subcloned into pBluescript SK- (Stratagene, La Jolla, CA) and sequenced. It was then cut out and subcloned into the XhoI/HindIII site of the pcDNA3-HA expression vector.

2.2.2 Histochemical Analyses

Immunohistochemistry and immunocytochemistry
Cultured cells and embryo sections were incubated with primary antibodies diluted in PBS over night at 4C and subsequenctly incubated with fluoresceinisothiocyanate (FITC; Dako), CY3 (Jackson), Alexa 546, Alexa 488 conjugated species specific antibodies, diluted in PBS for 1 hour at room temperature. For details on primary antisera and dilutions, see respective papers.

2.2.3 Immunoprecipitation

For immunoprecipitation, cells grown on 10 cm culture dish were extracted for 15 min in ice-cold lysis buffer (50 mM Tris/HCl (pH 7.4), 150 mM sodium chloride, 0.1% SDS, 1 mM EDTA, 1x protease inhibitor cocktail (Roche)). Then extracts were cleared by centrifugation at 15 000 X g for 5 min at 4C and stored at –80C until use. The extracts were incubated with anti HA antibody (Abcam) for 1 h in an ice bath. Immunoprecipitates were collected on Protein G Sepharose Fast flow beads (Amersham Biosciences) by overnight rotation, washed 4 times with lysis buffer, resuspended in 2x Laemmli sample buffer and subjected to SDS-PAGE followed by Western blot analysis using anti-Rac1 (Upstate) and anti-RhoA (Santa Cruz Biotechnology) antibodies. Western blotting was performed as described previously (Bryja et al., 2007).

2.2.4 Pull Down Assay

Glutathione-*S*-transferase (GST)-p21-activated kinase (PAK)-Cdc42/Rac interactive binding domain (CRIB), and GST-RHOtekin recombinant proteins coupled to sepharose-beads were prepared as described previously (Edlund et al., 2002, MBC 13(3):902-14). GST-PAK-CRIB, and GST-RHOtekin pull down assays for detection of activated RAC-1, CDC42 and RHO A, respectively were performed as follows. Cells were washed with ice cold PBS and subsequently allowed to lyse in ice cold lysis buffer (10 mM Tris-Cl – pH = 7.5, 110 mM NaCl, 1 mM EDTA, 10 mM $MgCl_2$, 1% Triton X-100, 0.1 % SDS, 20 mM β-glycerolphosphate, 1mM dithiotreitol, complete protease inhibitors) for 5 min. Crude cell lysates were spinned down in prechilled tubes at 14000 rpm for 5 min at 4°C. Supernatants (5% saved as input) were supplemented with bait proteins coupled to GST beads and tubes were incubated rotating end-over-end at 4°C for 15 min. Beads were washed 3 times with washing buffer (lysis buffer without SDS and protease inhibitors) on ice and subsequently mixed with 2x Laemmli buffer. Each sample was boiled for 5 minutes before loading on SDS-PAGE.

2.2.5 Wound Assay

Subconfluent C17.2 cells were transfected as above and then allowed to reach 100% confluency. The cells were then treated with 10μM Mitomycin C (Sigma-Aldrich) for 3 hours to arrest the cell cycle. Thereafter, a wound was made through the cells using a 200 μl pipet tip. Medium was changed to serum-reduced (1% FBS) to keep the cells from dividing, and a line was drawn underneath the culture dish perpendicular to the scratch. Pictures were taken just above or below the line under a light phase-contrast microscope, immediately and after 18 hours.

2.2.6 Aggregation Assay

MDCK cells were cultured until 70% confluency and then transfected with above mentioned plasmids using Lipofectamine 2000. After four hours the cells were carefully scraped off the culture dishes, washed in new medium, and counted. The cells were then transferred in drops of 30 μl (1000 cells/μl) to the inside of a 24-well plate-lid. With PBS in the bottom of the wells, the hanging drops were incubated for 24-48

hours to allow the cells to aggregate. The drops were then pipetted up and down 10 times with a 200 µl tip and analysed in a light microscope. For quantification representative micrographs from a single experiment were used. The pictures were overlaid with a 48 square grid. For each picture, the number of squares containing one or more aggregates with at least 4 cells was divided by the number of squares containing only single cells or aggregates with less than 4 cells. This yielded an aggregation index. The aggregation indexes from 4 micrographs per experimental condition was averaged and used for comparisons. A standard T-test was performed.

2.2.7 Proliferation and Apoptosis Labeling

Pregnant dams were given intraperitonic injections of BrdU (3 mg)4h before sacrifice. The embryos were then dissected out and treated as above (see Immunohistochemistry). The sections were then postfixed (4% paraformaldehyde, 15 min), PBS rinsed, and blocked with 3% H2O2 (10 min at 37-C). After a PBS rinse, sections were deproteinized in ice-cold 0.1 M HCl (20 min), DNA was denatured in 2 M HCl (30 min at 37-C), and pre-rinsed in 0.1 M Borax. The sections were then rinsed in PBS, blocked in 5% goat serum (Jackson Immunoresearch), 1% bovine serum albumin, and 0.05% Tween-20 in PBS for 30 min, followed by overnight incubation with monoclonal anti-BrdU (Sigma-Aldrich) in PBS with 1% bovine serum albumin and 0.05% Tween-20.

Sections were then washed in PBS. BrdU positive nuclei were visualized with a Mouse ExtraAvidin Peroxidase staining kit (Sigma-Aldrich) and Fast 3,3V-Diaminobenzidene tablet sets (Sigma-Aldrich). Sections were then dehydrated and mounted in Pertex (Histolab, Gothenburg). To study apoptosis, embryos were dissected and treated as above (see Immunohistochemistry). The ApopTag Peroxidase In Situ Apoptosis Detection Kit (Intergen, Purchase, NY) was used to detect apoptotic nuclei by the TUNEL method. The staining was performed according to the manufacturer's instructions.

2.3 CONFOCAL MICROSCOPE

Pictures were taken with a Leica TCS Confocal Microscope.

3 AIMS OF THE STUDY

The Wnts seem to have important, but as yet not clearly understood roles as determinants of cell proliferation, differentiation and maturation during both early and late phases of nervous system development. To further our understanding of this elusive yet interesting family of signaling factors, we studied transgenic mouse embryos that overexpress Wnt7a, or the Wnt PCP signaling protein Vangl2, in neuronal stem cells. We also studied loop-tail Vangl2 loss-of-function embryos, and cell lines transfected with expression constructs for Vangl2.

3.1 SPECIFIC AIMS OF THE PRESENT THESIS

3.1.1 The Roles of Wnt7a and Vangl2 in Cell-Cell Adhesion and Cell Migration

Evaluated by:

- analyzing different components of the cytoskeleton and adherence junctions i.e. actin, N-cadherin, β-catenin, Scrb1, Vangl2 (paper I and III)

- analyzing how the expression of *Rho* protein family; *Cdc42, Rac, and Rho* was affected (paper III)

- performing assays for cell-cell adherence (hanging drop cultures) and for cell migration (wound assays) (paper III)

3.1.2 The Effects of Wnt Signaling on Neuronal Differentiation

Evaluated by:

- analyzing the expression of β-tubulin III, a marker for undifferentiated neurons (paper II).

4 RESULTS AND DISCUSSION

4.1 WNT7A AND VANGL2 AFFECTS DISTRIBUTION OF CYTOSKELETAL COMPONENTS IN THE NEURAL TUBE (PAPER I AND III)

Overexpression of Wnt7a (paper I) and Vangl2 (paper III) in neuronal stem cells of mouse embryos changed the shape of the cytoskeleton, resulted in a disturbed neurulation and affected the rigidity of the embryos. In paper I we were originally aiming to study brain regionalization, but the phenotype of the Wnt7a overexpressing embryos surprised us. Based on the phenotype we started to investigate the actin cytoskeleton. Phalloidin staining indicated that actin over the adherence junctions was reduced or absent. This change in actin expression was also seen in Vangl2 overexpressing mouse embryos, and in loop-tail Vangl2 loss-of-function embryos.

4.1.1 Influence on the Adherens Junctions

Since the rigidity was affected we decided to investigate the expression of molecules involved in adherens junctions i.e. N-cadherin and β-catenin. Immunolabeling indicated reduction in the expression of N-cadherin and β-catenin parallel with actin levels in nestin-Wnt7a embryos. *N-cadherin* mRNA levels were however increased 2-fold in the neural tube. Our results suggest that N-cadherin levels are increased in the neural tube, but not in the adherens junctions, since focal presence of the N-cadherin protein at the adherens junctions was clearly lowered. Our conclusion is that *not* a reduction in *N-cadherin* mRNA levels, *but* a misslocalization of N-cadherin protein is the cause of the absence of N-cadherin in the adherens junctions. A mis-distribution of β-catenin and N-cadherin was also seen in the nestin-Vangl2 embryos and in loop-tail Vangl2 loss-of-function embryos (paper III).

4.1.2 The Role of Scrb1

In 2003 Mirreile Montcouquiol and collaborates identified Vangl2 and Scrb1 as PCP genes in mammals (Montcouquiol M., et al 2003). Since Wnt7a can signal through the PCP pathway we investigated the expression of Vangl2 (paper I) and Scrb1 (paper I and III) in our transgenic mouse embryos. We could see a significally higher expression of *Vangl2* mRNA in the neural tube of nestin-Wnt7a embryos. At the adherence junctions Scrb1 immunolabeling overlapped with actin and β-catenin staining. In the transgenic mouse embryos (paper I and III) Scrb1 labeling was highly reduced or non-detectable over the adherence junctions. We therefore conclude that the function of adherence junctions is regulated by the convergence of distinct Wnt signaling components, including PCP genes.

4.2 VANGL2 AFFECTS DISTRIBUTION OF CYTOSKELETAL COMPONENTS IN EPITHELIAL KIDNEY CELL LINES (PAPER III)

Two different epithelial cell lines, HEK293T and MDCK, were chosen in order to test the effect of Vangl2 overexpression on the actin cytoskeleton. Vangl2 was cloned into a pcDNA3 vector containing a HA-tag. We used an antibody against the HA-tag in order to visualize Vangl2. At the time when experiments were done no commercial antibody was available for Vangl2, which is why we used a tag to detect it. Staining with phalloidin revealed that intracellular actin deposits were common in HA-vangl2 transfected cells while we could rarely see this in control cells. Vangl2 overexpression also caused the cells to attach loosely to each other which made the process of immunolabeling quite hard, since many cells were lost during the washes.

We also tested actin homeostasis by co-transfecting our cells with a GFP-actin fusion protein together with a control plasmid or HA-Vangl2. The GFP tag was used to track the newly formed actin. GFP-actin was affected in a similar way as endogenous actin by Vangl2, but more intracellular actin deposits could be seen. The distribution of β-catenin and N-cadherin were investigated in HEK293T cells. Vangl2 transfection disrupted the expression pattern of β-catenin and N-cadherin in a similar way as seen in Wnt7a and Vangl2 overexpressing mouse embryos and in loop-tail Vangl2 loss-of-function embryos. We therefore concluded that Vangl2 gain-of-function in HEK293T cells interrups the expression of actin and its associated cytoskeletal components β-catenin and N-cadherin.

4.2.1 The Cellular Distribuation of Rac1 and P-JNK, but not RhoA is Altered (paper III)

In paper III we aimed to investigate the signaling pathways downstream of Vangl2. It is known that PCP signaling activates the small Rho-like GTPases RhoA and Rac1 (Fanto et al.,2000; Strutt et al., 1997), leading to JNK cascades (Fanto et al., 2000). Rac1 has been implicated in controlling adhesion between epithelial cells (Braga et al., 1997; Jou and Nelson, 1998), and affecting adherens junctions (Fischer and Quinlan, 1998; Quinlan, 1999).We could show that Rac1 labeling over adherence junctions was reduced or completely absent in Vangl2 overexpressing mouse embryos and in loop-tail Vangl2 loss-of-function embryos. The expression of active (phosphorylated) P-JNK, a downstream effector of Rac1, was also investigated. P-JNK expression was altered in our nestin-Vangl2 embryos. An abberant distribution of P-JNK could also be seen in loop-tails embryos.

The expression of Rac1 in HEK293T cells was investigated in a similar way as in embryos. Grain-like staining was found in the cytoplasm. Over the cell membranes Rac1 labeling was diffuse and its distribution was different from control transfected cells. RhoA labeling showed no significant difference between Vangl2 and control transfected cells. Neither could any significant difference of RhoA expression be seen between control and loop-tail mice. However, the expression of the downstream effector of Rac1, P-JNK, was altered and cytoplasmic labelling was highly reduced

compared to control transfected cells. Immunoprecipitation showed that Vangl2 binds to both Rac1 and RhoA, although a pull-down assay showed that their activity is not affected by Vangl2. Thus our findings show that Vangl2 affects Rac1 and P-JNK distribution but has very little effect on RhoA.

4.2.2 Vangl2 Overexpression Affects Cell Adhesion and Migration in Interaction with Rac1 (paper III)

Since we observed that overexpression of Vangl2 loosened the cells ability to attach, we investigated this further in an aggregation assay. For this experiment MDCK cells were used since they had a higher tendency to aggregate. The aggregation assay demonstrated that Vangl2 disrupts cell-cell adhesion. However cotransfection of Vangl2 with dominant negative Rac1 rescued this effect and led to normal level of cell aggregation. This was not observed when Vangl2 was cotransfected with dominant negative RhoA.

Reading a recent study (Wildenberg et al., 2006) made me realize that we should investigate whether Vangl2 affects cell migration. For this purpose we performed an aggregation assay. The experiments were done with C17.2 neural stem cells since they do not adhere to each other as our epithelial kidney cell lines do.We could show that Vangl2 increases cell migration. Dominant negative Rac1 was however able to block the effects of Vangl2 in cell migration. Knock-down of Rac1 interrupted cortical actin expression. This effect could be rescued when Vangl2 was cotransfected with Rac1RNAi. Together these results suggest that a balance between Rac1 and Vangl2 is essential for cell adhesion and mobility.

4.3 DIFFERENTIATION OF NEURONS IS AFFECTED WHEN WNT7A IS OVEREXPRESSED IN NEURAL STEM CELLS (PAPER II)

It's known that Wnts affects cell differentiation. We could also see that our Wnt7a embryos looked smaller and less developed than the controle embryos. We therefore decided to investigate the nestin-Wnt7a embryos level of neuronal differentiation. Differentiation of neurons was analyzed by investigating the expression of β-tubulin III, a marker for early differentiated neurons.

β-tubulin III expressing cells were labeled with the Tuj1 antibody. Quantification demonstrated that the proportion of β-tubulin III positive cells was significantly lower in the nestin-Wnt7a embryos than in their wild type littermates.

Wild type embryos investigated at E10.5 had high numbers of β-tubulin III positive cells mostly positioned in an ordered, ring-like pattern at the periphery of the neural tube, close to the pial surface. Their projections were parallel to the surface of the pial membranes . In the nestin-Wnt7a embryos β-tubulin III positive cells were present in a less ordered pattern, and they were more scattered medio-laterally. Their projections

were perpendicular to the neural tube walls, and the cells thereby resembled β-tubulin III positive cells in wild type E9.5 embryos. Thus, the pattern of β-tubulin III positive cells suggests a delay in neuronal development in the E10.5 nestinWnt7a embryos.

The expression of β-tubulin III positive cells in Vangl2 overexpressing mouse embryos were also investigated. Tuj1 expression was significantly lower in nestin-Vangl2 embryos compared to control embryos. Overexpression of Vangl2 in the neural stem cell line C17.2 yielded similar results (unpublished results). The decrease in β-tubulin III expression was paralleled by an increase in active β-catenin in the nuclei of Vangl2 overexpressing cells.

4.4 APOPTOSIS, BUT NOT PROLIFERATION WAS AFFECTED IN WNT7A OVEREXPRESSING EMBRYOS (PAPER I AND II)

Apoptosis was assayed by TUNEL staining. The ratio of TUNEL positive cells was significantly higher in the nestin-Wnt7a transgenics than in wild type embryos. Clusters of apoptopic nuclei were found in the roof and floor plate of the neural tube. Our finding suggests that apoptosis of neuroepithelial cells, in combination with disruption of the adherens junctions, resulted in the impaired neurulation.

Measurement of proliferation by BrdU injections did not reveal any change in E8.5 to E10.5 nestin-Wnt7a embryos (paper I). However, when we applied an alternative method, labeling with phosphohistone 3, we could se a slight reduction of proliferation in E9.5, but not E10.5 embryos (paper II).

We have also investigated the expression of Connexin43 in our nestin-Vangl2 mouse. The expression of Cx43 was significantly lowered compared to wild type embryos (unpublished results). Since Cx43 is a part of gap junction, it is possible that Vangl2 not only affects adherence junctions, but also is able to regulate gap junctions through Cx43.

5 CONCLUSIONS

This thesis describes the role of Wnt7a and its downstream signaling protein Vangl2 in generating a functional brain. We studied these effects by constructing transgenic mouse embryos that overexpress Wnt7a and Vangl2. These mouse embryos showed disturbed neurulation which resulted in an open neural tube. About 1 out of 1000 children are born with a defective neurulation. Since Vangl2 overexpressing mouse embryos showed the same phenotype as Wnt7a overexpressing mouse embryos and the fact that the distribution of actin, N-cadherin, Scrb1 and β-catenin was altered the same way in both embryos, we suggest in this thesis that the effect of Wnt7a overexpression on the cytoskeleton and adherence junctions is mediated via Vangl2.

Our results show that a balance between Rac1 and Vangl2 is essential for cell adhesion and mobility. Furthermore we could see that a loss of function of Rac1/Vangl2 and a gain of function of Rac1/Vangl2 leads to changes in actin cytoskeleton and disturbs adherens junctions.

Furthermore, our results indicate that the interaction of Vangl2 with Scrb1 may also contribute to migration and convergent extension movements. Combined, our results suggest that that the precise regulation of Vangl2 levels and its interaction with Rac1and Scrb1 is a key for the appropriate regulation of cell adhesion and migration.

We aim to continue our experiments by investigating whether Vangl2 effects the expression of active β-catenin. Our primary results indicate that this might be the case.

6 ACKNOWLEDGEMENTS

I would like to offer my deepest thanks and appreciation to all of you who supported and encouraged me during my doctoral education:

Thomas Ringstedt, my main supervisor, for his tremendous devotion to our research projects, exciting conversations and for teaching me all that there is to know about transgenic mice.

Hugo Lagercrantz, my co-supervisor, for graciously welcoming me into his laboratory group, introducing me to the field of pediatric research, and providing guidance throughout my research education.

Viveca Karlsson, Eva Lundberg and **Astrid Häggblad** for their kind assistance in logistical arrangements.

My "roommates" Jonas Berner, Yuri Shvarev, Linda Danielsson, Marco Bartocci, Jeo Park, Panos Papachristou, Zachi Horn and Johan Jäderstad, for filling the Brainstem and Lab rooms with laugther, and sharing your wisdom both in scientific and personal matters.

My family (mom and dad, my brother and sister-in-law and my beloved nephews) for teaching me the importance of exploration and for their unconditional support during this educational journey. Tack mamma för att jag kunde lätta mitt hjärta hos dig med jämna mellanrum! Telefonen har gått varm mellan Sverige och Finland!

My grandmother, for sharing my passion for books and my hunger for knowledge

My uncle, whose PhD defence set the direction of my life at the age of 13!

KI administration, for listening to us PhD students and for their constant work to create a better research environment. I'm greatful for all the help I have received from them in the preparation process of my thesis defence.

7 REFERENCES

Barkovich AJ.Neuroimaging of pediatric brain tumors. Neurosurg Clin N Am. 1992 Oct;3(4):739-69. Review.

Bhanot P, Brink M, Samos CH, Hsieh JC, Wang Y, Macke JP, Andrew D, Nathans J, Nusse R.A new member of the frizzled family from Drosophila functions as a Wingless receptor. Nature. 1996 Jul 18;382(6588):225-30.

Bryja, V., Schulte, G., Rawal, N., Grahn, A., and Arenas, E. (2007). Wnt-5a induces Dishevelled phosphorylation and dopaminergic differentiation via a CK1-dependent mechanism. J Cell Sci *120*, 586-595.

Cadigan KM, Nusse R.Wnt signaling: a common theme in animal development. Genes Dev. 1997 Dec 15;11(24):3286-305. Review

Chenn A, Walsh CA.Regulation of cerebral cortical size by control of cell cycle exit in neural precursors.Science. 2002 Jul 19;297(5580):365-9.

Castelo-Branco, G.,Wagner, J., Rodriguez,F.J., Kele, J., Sousa, K., Rawal, N., Pasolli, H.A., Fuchs, E., Kitajewski, J., Arenas, E., 2003. Differential regulation of midbrain dopaminergic neuron development by Wnt-1, Wnt-3a, and Wnt-5a. Proc. Natl. Acad. Sci. U. S. A. 100, 12747 – 12752.

Deardorff, M.A., Tan, C., Conrad, L.J., Klein, P.S., 1998. Frizzled-8 is expressed in the Spemann organizer and plays a role in early morphogenesis. Development 125, 2687 – 2700.

Dickinson, M.E., Krumlauf, R., McMahon, A.P., 1994. Evidence for a mitogenic effect of Wnt-1 in the developing mammalian central nervous system. Development 120, 1453 – 1471.

Dorsky RI, Moon RT, Raible DW.Control of neural crest cell fate by the Wnt signaling pathway. Nature. 1998 Nov 26;396(6709):370-3.

Fanto M, Weber U, Strutt DI, Mlodzik M.Nuclear signaling by Rac and Rho GTPases is required in the establishment of epithelial planar polarity in the Drosophila eye. Curr Biol. 2000 Aug 24;10(16):979-88.

Fraser SE, Bronner-Fraser M.Migrating neural crest cells in the trunk of the avian embryo are multipotent. Development. 1991 Aug;112(4):913-20.

Gunhaga, L., Marklund, M., Sjodal, M., Hsieh, J.C., Jessell, T.M., Edlund, T., 2003. Specification of dorsal telencephalic character by sequential Wnt and FGF signaling. Nat. Neurosci. 6, 701 – 707.

Hackett DA, Smith JL, Schoenwolf GC. 1997 Epidermal ectoderm is required for full elevation and for convergence during bending of the avian neural plate. Dev Dyn. Dec;210(4):397-406.

Hall, A.C., Lucas, F.R., Salinas, P.C., 2000. Axonal remodeling and synaptic differentiation in the cerebellum is regulated by WNT-7a signaling. Cell 100, 525 – 535.

Hemmati-Brivanlou A, Melton DA.Inhibition of activin receptor signaling promotes neuralization in Xenopus. Cell. 1994 Apr 22;77(2):273-81.

Hemmati-Brivanlou A, Kelly OG, Melton DA.Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity.Cell. 1994 Apr 22;77(2):283-95.

Hume, C.R., Dodd, J., 1993. Cwnt-8C: a novel Wnt gene with a potential role in primitive streak formation and hindbrain organization. Development 119, 1147 –1160

Heisenberg, C.P., Tada, M., Rauch, G.J., Saude, L., Concha, M.L., Geisler, R., Stemple, D.L., Smith, J.C., Wilson, S.W., 2000. Silberblick/Wnt11 mediates convergent extension movements during zebrafish gastrulation. Nature 405, 76 – 81.

Hildebrand, J.D., Soriano, P., 1999. Shroom, a PDZ domain-containing actin-binding protein, is required for neural tube morphogenesis in mice. Cell 99, 485 – 497.

Hirabayashi, Y., Itoh, Y., Tabata, H., Nakajima, K., Akiyama, T., Masuyama, N., Gotoh, Y., 2004. The Wnt/{beta}-catenin pathway directs neuronal differentiation of cortical neural precursor cells. Development 131, 2791 – 2801.

Huelsken, J., Birchmeier, W., 2001. New aspects of Wnt signaling pathways in higher vertebrates. Curr. Opin. Genet. Dev. 11, 547 – 553.

Jou TS, Schneeberger EE, Nelson WJ.1998 Structural and functional regulation of tight junctions by RhoA and Rac1 small GTPases. J Cell Biol. Jul 13;142(1):101-15.

Krylova O, Herreros J, Cleverley KE, Ehler E, Henriquez JP, Hughes SM, Salinas PC.2002 WNT-3, expressed by motoneurons, regulates terminal arborization of neurotrophin-3-responsive spinal sensory neurons. Neuron. Sep 12;35(6):1043-56

Letamendia A, Labbé E, Attisano L. 2001Transcriptional regulation by Smads: crosstalk between the TGF-beta and Wnt pathways. J Bone Joint Surg Am. ;83-A Suppl 1(Pt 1):S31-9.

Lee, H.Y., Kleber, M., Hari, L., Brault, V., Suter, U., Taketo, M.M., Kemler, R., Sommer, L., 2004. Instructive role of Wnt/beta-catenin in sensory fate specification in neural crest stem cells. Science 303, 1020 – 1023.

Lothian, C., Lendahl, U., 1997. An evolutionarily conserved region in the second intron of the human nestin gene directs gene expression to CNS progenitor cells and to early neural crest cells. Eur. J. Neurosci. 9, 452 – 462.

Lu, C.C., Robertson, E.J., Brennan, J., 2004a. The mouse frizzled 8 receptor is expressed in anterior organizer tissues. Gene Expr. Patterns 4, 569 – 572.

Lu, X., Borchers, A.G., Jolicoeur, C., Rayburn, H., Baker, J.C., Tessier-Lavigne, M., 2004b. PTK7/CCK-4 is a novel regulator of planar cell polarity in vertebrates. Nature 430, 93 – 98.

Lumsden A, Krumlauf R. 1996 Patterning the vertebrate neuraxis. Science. Nov15;274(5290):1109-15. Review.

Megason SG, McMahon AP 2002 A mitogen gradient of dorsal midline Wnts organizes growth in the CNS. Development. May;129(9):2087-98.

Montcouquiol, M., Rachel, R.A., Lanford, P.J., Copeland, N.G., Jenkins, N.A., Kelley, M.W., 2003. Identification of Vangl2 and Scrb1 as planar polarity genes in mammals. Nature 423, 173 – 177.

Montcouquiol M, Sans N, Huss D, Kach J, Dickman JD, Forge A, Rachel RA, Copeland NG, Jenkins NA, Bogani D, Murdoch J, Warchol ME, Wenthold RJ, Kelley MW.2006Asymmetric localization of Vangl2 and Fz3 indicate novel mechanisms for planar cell polarity in mammals. J Neurosci. May 10;26(19):5265-75

Nordstrom, U., Jessell, T.M., Edlund, T., 2002. Progressive induction of caudal neural character by graded Wnt signaling. Nat. Neurosci. 5, 525 – 532.

Nüsslein-Volhard C, Lohs-Schardin M, Sander K, 1980 Cremer C.A dorso-ventral shift of embryonic primordia in a new maternal-effect mutant of Drosophila. Nature. Jan 31;283(5746):474-6. No abstract available.

Nüsslein-Volhard C, 1980Wieschaus E.Mutations affecting segment number and polarity in Drosophila.Nature. Oct 30;287(5785):795-801.

Patapoutian A, Reichardt LF.Roles of Wnt proteins in neural development and maintenance. Curr Opin Neurobiol. 2000 Jun;10(3):392-9. Review.

Rakic P. 1971Guidance of neurons migrating to the fetal monkey neocortex. Brain Res. Oct 29;33(2):471-6.

Rubenstein JL, Beachy PA1998 Patterning of the embryonic forebrain. Curr Opin Neurobiol. Feb;8(1):18-26.

Saneyoshi T, Kume S, Amasaki Y, Mikoshiba K. 2002 The Wnt/calcium pathway activates NF-AT and promotes ventral cell fate in Xenopus embryos.Nature. May 16;417(6886):295-9.

Serbedzija GN, Bronner-Fraser M, Fraser SE.A vital dye analysis of the timing and pathways of avian trunk neural crest cell migration. Development. 1989 Aug;106(4):809-16

Schambony A, Wedlich D.2007 Wnt-5A/Ror2 regulate expression of XPAPC through an alternative noncanonical signaling pathway.Dev Cell. May;12(5):779-92.

Strutt DI, Weber U, Mlodzik M.1997 The role of RhoA in tissue polarity and Frizzled signaling. Nature. May 15;387(6630):292-5.

Veeman, M.T., Axelrod, J.D., Moon, R.T., 2003a. A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. Dev. Cell 5, 367 – 377.

Veeman, M.T., Slusarski, D.C., Kaykas, A., Louie, S.H., Moon, R.T., 2003b. Zebrafish prickle, a modulator of noncanonical Wnt/Fz signaling, regulates gastrulation movements. Curr. Biol. 13, 680 – 685.

Wallingford, J.B., Rowning, B.A., Vogeli, K.M., Rothbacher, U., Fraser, S.E., Harland, R.M., 2000. Dishevelled controls cell polarity during Xenopus gastrulation. Nature 405, 81 – 85.

Wilson, S.I., Rydstrom, A., Trimborn, T., Willert, K., Nusse, R., Jessell, T.M., Edlund, T., 2001. The status of Wnt signaling regulates neural and epidermal fates in the chick embryo. Nature 411, 325 – 330.

Willert, K., Brown, J.D., Danenberg, E., Duncan, A.W., Weissman, I.L., Reya, T., Yates, J.R., Nusse, R., 2003. Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature 423, 448 – 452.

Wong LL, Adler PN.1993 Tissue polarity genes of Drosophila regulate the subcellular location for prehair initiation in pupal wing cells. J Cell Biol. Oct;123(1):209-21

Ybot-Gonzalez, P., Copp, A.J., 1999. Bending of the neural plate during mouse spinal neurulation is independent of actin microfilaments. Dev. Dyn. 215, 273 – 283.