THE ROLE OF WNT SIGNALING IN TEMPORAL PATTERNING AND CELL FATE SPECIFICATION OF THE MIDBRAIN HINDBRAIN DOMAIN

A Dissertation

by

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ABSTRACT

The Midbrain Hindbrain Domain (MHD) is a region of the central nervous system consisting of the midbrain, midbrain hindbrain boundary (MHB) and anterior hindbrain. The MHB is an organizer that patterns the midbrain and anterior hindbrain. It is important to understand how organizers such as the MHB are formed to better understand how the vertebrate brain is patterned and develops. I have examined how timing of signaling by the canonical Wnt/β -catenin signaling pathway affects MHD specification and patterning by inhibiting Wnt signaling at specific developmental time intervals and analyzing the resulting phenotypes by morphological analysis and in situ hybridization.

Four Wnt genes, *wnt1*, *wnt10b*, *wnt3*, and *wnt3a*, have overlapping expression patterns in the MHD, but their relative roles in MHB development are unclear. *wnt3a*, *wnt1*, and *wnt10b* are activated earlier in development than *wnt3*. *wnt3a*, *wnt1*, and *wnt10b* have been shown to work in combination to regulate MHB development which lead to the question what is the role of *wnt3* in this process. I specifically examined the role of *wnt3* during MHB development by knocking down *wnt3* in combination with the other three Wnt genes and performed in situ hybridizations using anterior and posterior neural markers.

We observed that after inhibition of Wnt signaling at early developmental time intervals, midbrain and anterior hindbrain formation is disrupted but not MHB development. When Wnt signaling is blocked at later developmental time intervals,

MHD patterning and formation are disrupted. Our data show forebrain fates expand posteriorly and midbrain and anterior hindbrain fates are reduced at later developmental stages. These data suggest Wnt signaling represses anterior neural fates and advances posterior neural fates in MHD patterning and specification.

When *wnt3* is inhibited in combination with *wnt1* and *wnt10b,* I observed that the MHB is partially reduced, whereas the MHB is absent when *wnt3* and *wnt3a* in combination with *wnt1* and *wnt10b* are inhibited. When *wnt3* and *wnt3a* are blocked, the MHB is absent. These data suggest that these Wnt genes work in combination to regulate MHB formation but have differential requirements in this process.

In conclusion, our results suggest Wnt signaling represses forebrain fates, while promoting midbrain, MHB, and anterior hindbrain. Also, multiple Wnt genes expressed in the MHB regulate MHB formation, but their functions are different.

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NOMENCLATURE

A/P	Anterior Posterior
APC	Adenomatosis Polyposis Coli
AVE	Anterior Visceral Endoderm
BMP	Bone Morphogenetic Protein
CK1	Casein Kinase 1
CNS	Central Nervous System
FGF	Fibroblast Growth Factor
Fz	Frizzled
GSK3	Glycogen Synthase Kinase 3
GRN	Gene Regulatory Network
HPF	Hours Post Fertilization
LEF	Lymphoid Enhancer-Binding Factor
LRP	LDL receptor related protein
MDO	Middiencephalic Organizer
Mes-R1	mesencephalon-rhombomere 1
MHB	Midbrain Hindbrain Boundary
MHD	Midbrain Hindbrain Domain
MO	Morpholino
TCF	T Cell Factor
R1	Rhombomere 1

ZLI	Zona Limitans Intrathalamica

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CHAPTER I

INTRODUCTION

The central nervous system is patterned along its anterior-posterior axis into the forebrain, midbrain, hindbrain, and spinal cord, but patterning also establishes subregions, such as the Midbrain Hindbrain Domain (MHD), which consists of the midbrain, midbrain hindbrain boundary (MHB), and anterior hindbrain. A fundamental question in developmental biology is how induction and anteroposterior patterning of the central nervous system occurs in vertebrates.

The MHD Is a Region of the Central Nervous System

The central nervous system is subdivided along its anterior-posterior axis forming specific structures. The neural tube starts as an elongated tube, which bulges out in specific locations to forms vesicles. The first vesicle is the prosencephalon, forming the forebrain primordium, the second is the mesencephalon, forming the midbrain primordium, and the third is the rhombencephalon, forming the hindbrain primordium (Gilbert, 2010). After this first regionalization, the neural tube is further subdivided into more discrete regions. The prosencephalon is subdivided into the telencephalon and diencephalon, and the rhombencephalon is subdivided into the metencephalon and myelencephalon (Gilbert, 2010). The rhombencephalon forms eight domains called rhombomeres, the first rhombomere, r1, develops into the anterior hindbrain. In mice the area spanning from the mesencephalon to r1 is named the mes-r1 domain, while in zebrafish this region is named the MHD. The MHD consists of the mesencephalon or

midbrain, MHB, and anterior hindbrain. The question of how the central nervous system is induced and regionalized is a question that has been examined for many years.

The Organizer: The Beginning of Understanding Neural Induction

In the 1920s, Hans Spemann and Hilde Mangold transplanted the dorsal blastopore lip of a gastrula stage donor embryo into the ventral side of a gastrula stage host embryo in salamanders. A secondary body axis including the notochord was induced (Spemann and Mangold, 1923). The dorsal blastopore lip was termed the organizer because it induced surrounding tissue fated to be epidermis to become neural (Spemann and Mangold, 1923). Neural induction is when tissue is specified to adopt neural fate (Wilson and Edlund, 2001). Another organizer in the central nervous system is the MHB. Understanding how organizers such as the MHB form helps us to better understand how central nervous system induction and patterning occurs. A question that these experiments did not answer was how does regionalization of the central nervous system occurs.

Regionalization of the Central Nervous System

A hypothesis to explain how the central nervous system is regionalized proposed that factors along the anterior posterior axis induce specific structures. Otto Mangold transplanted different portions of late gastrula stage archenteron roof, or the roof of the primitive gut, along the anterior posterior axis into early gastrula stage blastocoels in newts (Gilbert, 2010). The most anterior transplanted portion induced a head like region. Tissue transplanted from more posterior regions along the axis induced more posterior fates. Tissue transplanted from the most posterior portion of the archenteron induced a

tail and trunk region (Gilbert, 2010). These experiments suggested that factors along the anterior-posterior axis induce specific structures, which regionalize the central nervous system (Gilbert, 2010). More transplantation experiments were performed to examine if developmental timing plays a role in neural induction.

Timing Is Important for Neural Induction

Mangold examined the role of timing in neural induction by performing transplantation experiments in salamanders. Transplantation of a young gastrula stage dorsal blastopore lip from a donor embryo into a gastrula stage host embryo induces a head. Transplantation of a late gastrula stage donor blastopore lip from a donor embryo into a gastrula stage host induces a tail and trunk (Gilbert, 2010). These data suggest cells from an early staged organizer produce anterior structures, while later staged cells from the organizer produce more posterior structures (Gilbert, 2010). These experiments show timing is an important factor in neural induction. These and other experiments led to models proposing how neural posteriorization occurs.

Neural Posteriorization Transforms Tissues into the MHD

Neural induction explains how neural tissue is induced and specified, but does not propose how more posterior structures such as the midbrain and anterior hindbrain are induced. Neural posteriorization explains how induction posterior neural cell fates occur. Two prevailing models propose how posterior neural tissue is induced.

In the 1950s, Nieuwkoop proposed the Activation-Transformation model. This model states induction of posterior neural cell fates occurs in two phases. First, a signaling pathways or activation signal, specifies forebrain cell fates. Second, another

signaling pathway or transformation signal induces forebrain to become posterior cell fates such as the midbrain, hindbrain and spinal cord (Nieuwkoop, 1952). Saxèn and Toivinen proposed another model to explain anterior posterior axis patterning of the neural plate.

Saxèn and Toivinen proposed a double gradient model to explain patterning of the anterior posterior neural plate. They proposed a gradient acts on mesoderm tissue and another gradient acts on neural tissues. These two gradients intersect to pattern the anterior posterior axis of the neural plate (Saxèn, 2001). These models are not mutually exclusive and experiments over the years support both models. These models both propose signaling pathways acting as morphogens in neural posteriorization.

Morphogens Activate Genes through a Gradient

Morphogens are signaling molecules that act over distances to activate cellular responses that pattern tissue (Rogers and Schier, 2011). Morphogens are proposed to activate genes through a concentration gradient that forms as the signaling molecules spread throughout surrounding tissue from a localized source. The gradient of signal acts on cells in a concentration dependent manner to specify cell fates through differential gene expression (Ashe and Briscoe, 2016).

Multiple models propose how cells interpret morphogen concentration gradients. A morphogen gradient can be interpreted as a step gradient. The morphogen may need to reach a certain threshold in order for cells to respond to its signal (Ashe and Briscoe, 2016). The temporal integration model proposes how long the cells are exposed to the morphogen affects their response. Studies show a correlation in the spatial activation of

genes with the time and potency of morphogen (Ashe and Briscoe, 2016). A study in which prospective forebrain explants from chick embryos were cultured with or without Wnt ligand in the culture medium showed that Wnts act in a graded manner to posteriorize neural tissue. When prospective forebrain explants were cultured without any Wnt ligand, forebrain markers were expressed in the explants (Nordstrom et al., 2002). When prospective forebrain explants were cultured with low concentrations of Wnt ligands, anterior and posterior forebrain markers were expressed. As the prospective forebrain explants were cultured with increasing concentrations of Wnt ligand, more midbrain and hindbrain markers were expressed. These experiments suggest Wnt ligands act as a gradient in posteriorization of the neural plate (Nordstrom et al., 2002). A possible interpretation for cell responses to the Wnt ligand is that cells in the posterior neural plate are exposed to higher concentrations of Wnt, but an additional possibility is that they are exposed for a longer period of time, thereby allowing these cells to adopt a more posterior fate like the midbrain or hindbrain. Other pathways, such as BMP, FGF, and retinoic acid, are proposed to act as morphogens and are involved in neural induction.

Signaling Pathways Regulating Both Neural Induction and Posteriorization

BMP plays a role in specifying cells to become mesodermal during embryogenesis, while inhibition of BMP specifies cells to become ectoderm (Wang et al., 2014; Levine and Brivanlou, 2007). The BMP pathway is activated when a BMP ligand binds to type I and type II serine/threonine kinase receptors, forming a complex that activates downstream signaling. The type II receptor is phosphorylated and in turns

phosphorylates the type I receptor. The type I receptor phosphorylates R-Smads 1/5/8, which are receptor regulated Smads. A complex of Smad proteins is formed when R-Smads 1/5/8 associate with the co-mediator Smad, Smad4. This complex of Smad proteins enters into the nucleus where it acts as a transcription factor and interacts with corepressors and coactivators in regulating gene expression (Rahman et al., 2015; Wang et al., 2014).

FGF is involved in many developmental processes including cell migration, differentiation, and proliferation (Turner and Grose, 2010). FGF signaling transduction is activated when a FGF ligand binds to FGF receptors causing the receptors to dimerize. Kinase domains on the receptors phosphorylate each other allowing for the recruitment of adaptor protein and activating downstream signaling (Turner and Grose, 2010). Studies show that FGF and Wnt signaling pathways act in parallel in many developmental processes including MHB formation (Olander et al., 2006).

The Canonical Wnt pathway is involved in many developmental processes. There are two receptors involved in the transduction of Wnt signaling: Fizzled (Fz), a seven-pass membrane receptor and the LDL receptor-Related Lipoprotien (LRP), a single pass transmembrane receptor. In humans there are 19 Wnt ligands and 10 Fz receptors, and two LRP co-receptors, LRP5 and LRP6 (Niehrs, 2012). How different Fz receptors and LRP receptors interact with Wnt ligands is not well understood (Niehrs, 2012). Thus, how different combinations of receptor and ligand interactions are related to signaling specificity is an open question. When the Wnt pathway is not activated by a Wnt ligand, a destruction complex consisting of APC, Axin, and the kinases GSK3 and Ck1, degrade

the cytoplasmic protein, b-catenin. In the absence of Wnt signals, b-catenin is unable to enter the nucleus and activate Wnt target genes (Clevers and Nusse, 2012). When a Wnt ligand binds to the Fz in the presence of the LRP coreceptor, the destruction complex does not form, allowing b-catenin to be stabilized and enters the nucleus, where it displaces Groucho, a transcriptional repressor, and activates Wnt target genes by binding to Wnt effectors, TCF/LEF (Clevers and Nusse, 2012). Studies show Wnt along with FGF and retinoic acid play a role in anterior posterior patterning of the neural plate (Kudoh et al., 2002).

Retinoic acid patterns the hindbrain during embryonic development (Rhinn and Dollé, 2012). Retinoic acid regulates gene expression by forming a complex with retinoic acid receptors and retinoid X receptors, which in turn binds to RA-response elements in target genes. A conformational change occurs in the binding domain, which releases co-repressors, and recruits co-activators that in turn activate target gene transcription (Rhinn and Dollé, 2012). Studies show all three of these pathways regulate posteriorization of the neural plate.

FGF, WNT, Retinoic Acid Posteriorize the Neural Plate

Three molecules, retinoic acid, FGF, and Wnt, were discovered to act as posteriorizing agents in anterior-posterior patterning of the neural plate. Xenopus embryos treated with retinoic acid lack anterior neural structure and induce more posterior structures (Durston et al., 1989). Using Xenopus explants, experiments showed bFGF could induce posterior markers of the hindbrain and spinal cord (Cox and Hemmati-Brivanlou, 1995). In Xenopus, when animal caps are exposed to a Wnt ligand,

more posterior neural markers are expressed and there is a reduction in expression of anterior neural markers (McGrew et al., 1997). While these pathways are involved in neural posteriorization, another signaling pathway, BMP, acts to initiate formation of the central nervous system.

Neural Induction Starts the Formation of the Central Nervous System

Neural induction begins the process of forming neural tissues. The default model proposes a progressive process of how neural induction occurs. This model states *chordin*, *follistatin* and *noggin*, which are expressed in the dorsal organizer, antagonize BMP, which induces neural fates (Muñoz-Sanjáun and Brivanlou, 2002). The default model was proposed based on studies performed in Xenopus, but is this model conserve in other vertebrates?

According to the default model, in amphibians, neural induction and differentiation occur during gastrulation when BMP antagonist are secreted by the organizer; however, studies in chick embryos show that neural cell specification begins before gastrulation and that the node (or organizer) is not needed in neural induction (Wilson and Edlund, 2001). In chick, these neural precursor cells are committed to neural fates at the end of gastrulation (Wilson and Edlund, 2001). In both avian and mice, studies show that, unlike Xenopus, inhibiting BMP does not produce neural fates but can favor epidermal fates over neural fates (Wilson and Edlund, 2001).

In mouse, the BMP antagonist, *follistatin,* is not expressed in the node; however, like amphibians, studies in mammalian cell lines show BMP antagonists are sufficient for induction of neural fates (Muñoz-Sanjáun and Brivanlou, 2002). When either

follistatin or *noggin* are knocked out in mouse, neural tissue is formed, suggesting these genes are not required for neural induction (Muñoz-Sanjáun and Brivanlou, 2002). While there are discrepancies in the default model in between Xenopus, chick, and mouse, in zebrafish, BMP antagonists in the organizer are needed for neural fate specification (Appel, 2000; Wilson and Edlund, 2001). After neural tissue is induced a neural tube is produced.

Primary and Secondary Neurulation Form the Neural Tube Setting up Regionalization of the MHD

Primary neurulation and secondary neurulation form the neural tube with the primary neurulation forming the anterior neural tube and secondary neurulation forming the posterior neural tube (Lowry and Sive, 2004). The process of primary neurulation is similar across vertebrates and occurs in four steps.

First, ectodermal cells receive signals from the dorsal mesoderm and elongate. Convergence extension movements shape and lengthen the neural plate along its anterior posterior axis (Smith and Schoenwolf, 1997). The neural plate bends and folds in the middle forming a medial hinge point (Smith and Schoenwolf, 1997). The cells at this hinge point attach to the notochord below, forming a groove at the midline by reducing the height of the cells. Two more grooves called the dorsolateral hinge points form from the rest of the ectoderm. Neural folds form through the shaping of the neural plate by convergence extension and folding (Smith and Schoenwolf, 1997).

The last step in the formation of the neural tube is when it closes. This occurs when the folds come together and attach to one another (Smith and Schoenwolf, 1997). After primary neurulation occurs, secondary neurulation begins.

In secondary neurulation, the medullary cord is formed from mesenchyme cells from the ectoderm and endoderm (Lowry and Sive, 2004). The medullary cord undergoes a mesenchymal-to-epithelial transition forming the lumen and a neural tube (Lowry and Sive, 2004).

Anterior Posterior Patterning of the Neural Plate Occurs through Morphogen Gradients

Studies show that the BMP inhibitors, *chordin*, *noggin*, *follistatin*, and the Wnt inhibitors, *cerberus, dickkopf, frzb*, and IGF block these pathways, which induces formation of head and brain (Gilbert, 2010). Posterior structures such as the truck are formed by gradients of Xwnt8 and retinoic acid, which are expressed at higher concentrations in the posterior neural plate. This Wnt signaling gradient works through Fgf signaling indirectly to posteriorize the neural plate (Domingos et al., 2001). These signaling pathways are conserved in the process patterning the anterior posterior axis of the neural plate.

In zebrafish, Wnt, retinoic acid, and FGF are needed for patterning the anterior posterior neural plate. Wnt signaling inhibits anterior neural genes and then FGF, retinoic acid and Wnt initiate posterior neural genes (Kudoh et al., 2002).

In chick embryos, FGF, Wnt and retinoic acid interact in patterning the anterior posterior axis of the neural plate. FGF signaling from the primitive streak blocks retinoic

acid synthesizing enzymes and receptors that inhibits retinoic acid from posteriorizing the paraxial mesoderm. FGF promotes Wnt signaling which in turn promotes retinoic acid signaling in the presomitic mesoderm, which extends the anterior posterior axis (Gilbert, 2010).

Mouse embryos have two organizers: one is the node that acts similarly to the organizer in amphibians, and the other is the anterior visceral endoderm (AVE), which is similar to the hypoblast in chicks (Gilbert, 2010). Lefty-1 and Cerebus, which inhibit Nodal signaling, are secreted from the AVE forming an anterior region that renders Nodal signaling ineffective. Wnt and Nodal in the AVE suppress the primitive streak, which allows formation of more posterior structures (Gilbert, 2010). Similar to chick embryos, there is a gradient of BMP, FGF and Wnt at higher concentrations in the posterior neural plate and absent in the anterior neural plate (Gilbert, 2010). In the anterior portion of the embryo are large concentrations of Wnt and BMP antagonists, preventing these signaling pathways from specifying posterior neural fates. At later stages in gastrulation a gradient of retinoic acid is produced with higher concentrations in the posterior and absent in the anterior portions of the embryo. Retinoic acid posteriorizes the embryo by activating Hox genes (Gilbert, 2010). Not only are the pathways regulating posteriorization of the neural plate conserved in vertebrates, but the genes regulating MHD formation are also similar.

Genes Regulating MHD Patterning are Conserved in Mouse and Chick

Studies in multiple vertebrate model organisms show the genetic regulation of MHD development is conserved. The first studies examining MHD formation were

chick transplantation experiments. Early gastrula stage quail mesencephalon was transplanted into chick mesencephalon resulting in mesencephalon tissue. When mesencephalon explants were transplanted into caudal prosencephalon, mesencephalon tissue was produced (Martinez et al., 1991). Transplantation experiments in which mesencephalon or metencephalon from chicks were placed into similar area in quails showed the development of cerebellar tissues. These experiments were repeated but quail tissue was transplanted into chicks, yet yielded the same results (Martinez et al., 1991). Studies were performed in multiple vertebrate model organisms to examine the possible genes involved in regulating MHD development.

otx2, is expressed in the presumptive midbrain and *gbx2* (*gbx1* in zebrafish), is expressed in the presumptive hindbrain. *pax2a* (formerly *pax2* and *pax2.1*) overlaps with both of these genes. *fgf8a* overlaps with *gbx2,* while *wnt1* overlaps *otx2* (Zervas et al., 2005). The expression patterns of *wnt1, fgf8, pax2, otx2, and gbx2* are conserved in vertebrates. To better understand the functional role of genes expressed in the MHD, gain-of-function experiments were performed.

Gain-of-function studies in chick and mouse examined the function of genes expressed in the MHD. *otx2* overexpression shows an increase in *wnt1* and decrease in *gbx2* expression, while *gbx2* overexpression demonstrates a decrease in both *otx2* and *wnt1* (Zervas et al., 2005). Gain-of-function experiments with *pax2a* demonstrate an increase in *fgf8a* expression. *wnt1* overexpression shows an increase in *fgf8a* expression, while *fgf8a* overexpression shows an increase in *wnt1, gbx2* and *eng2a* expression and a

decrease in *otx2* expression (Zervas et al., 2005). Loss-of-function experiments were performed to further examine theses genes function in MHD formation

Loss-of-function experiments in mouse examine how multiple genes expressed in the MHD regulate its development. Deletion of *otx2* causes a loss of midbrain, while a deletion of *gbx2* causes a loss of hindbrain (Zervas et al., 2005). When *pax2a* is deleted, posterior midbrain is absent and deletion of *eng2a* (formerly *en2*), which is expressed in the MHD, causes a slight reduction in anterior hindbrain (Zervas et al., 2005). In mice that lack *wnt1*, there is a deletion of midbrain, MHB, and anterior hindbrain (Danielian and McMahon, 1996). A deletion of *fgf8a* produced a deletion of both midbrain and anterior hindbrain (Zervas et al., 2005).

Multiple Wnt Genes are Expressed in the MHD

In vertebrates there are multiple Wnt genes expressed in the MHD. In the mouse, *wnt3, wnt3a*, and *wnt1* are expressed in the forebrain and midbrain (Roelink and Nusse 1991; Ikeya et al., 1997). Deletion of both *wnt1* and *wnt3a* causes a reduction in the hindbrain (Ikeya et al., 1997). In chicks, *wnt3a* is expressed in the midbrain and hindbrain, while *wnt3* is expressed in the forebrain, midbrain, and hindbrain (Holladay et al., 1995; Robertson et al., 2004). In chicks, *wnt1* and *wnt3a* are needed in proliferation during spinal cord development (Megason and McMahon, 2002).

wnt3a in Xenopus is expressed in forebrain and midbrain (Wolda et al., 1992). In animal caps, Wnt3a overexpression, shows a decrease in posterior neural markers and increase in anterior neural markers (Dibner et al., 2001). In zebrafish, *wnt3* and *wnt3a* are expressed in the MHB and anterior hindbrain and both these genes are involved in

forebrain development (Mattes et al., 2012). Studies show that *wnt1* and *wnt10b* are expressed in the midbrain and MHB, and when these genes are deleted, the MHB is reduced at 24 hpf (Lekven et al., 2002). When *wnt3a, wnt1*, and *wnt10b* are inhibited the MHB is absent at 24 hpf, suggesting Wnt genes work in combination to regulate MHB development (Buckles et al., 2004).

MHB Formation Occurs in Three Phases

Studies show the genetic regulation of MHB development is similar in vertebrates. In the positioning phase, *wnt8a* (formerly *wnt8*) activates *otx2* and represses *gbx1* expression to position the MHB. *otx2* and *gbx1* are transcription factors that are activated at 6 hpf (Rhinn et al., 2005). *otx2* expression in the presumptive midbrain and *gbx1* expression in the presumptive hindbrain overlap. The MHB is positioned between these two genes expression patterns (Rhinn et al., 2005). In mice, *gbx2* expression is shifted anteriorly in *otx2* mutants, while *otx2* is expanded posteriorly in *gbx2* mutants suggesting these genes are needed to position the MHB (Li and Joyner, 2001). After the MHB is positioned, the MHB GRN is activated and initiates MHB formation (Fig. 1).

pax2a, which is a transcription factor, and *fgf8a* and *wnt1*, which are signaling molecules, are activated between 8-9 hpf and form a GRN, which specifies MHB cell fates (Raible and Brand, 2004). It is unknown how these genes are activated, though studies show that when one of these genes is deleted; the other two genes' activation is not affected (Raible and Brand, 2004). This phase of MHB development is called the establishment phase and overlaps with the maintenance phase.

The third phase is called the maintenance phase. In this phase, *pax2a*, *fgf8a*, and *wnt1* form an auto-regulatory loop beginning at about 12 hpf, which maintains the development of the MHB. Studies show that when one of these genes is deleted, the other two genes' expression is not maintained (Raible and Brand, 2004). In mice, when *wnt1* is deleted, *eng2a* expression is initiated but becomes reduced over time (Danielian and McMahon, 1996). In zebrafish *pax2a* mutants, expression of *wnt1* and *eng2a* are activated but decrease during development (Lun and Brand, 1998). Interestingly, in these mutants, *fgf8a* expression is normal in the MHB. These data suggest *pax2a* is needed to maintain expression of *wnt1* and *eng2a* during MHB development but not *fgf8a* (Lun and Brand, 1998). In zebrafish *fgf8a* mutants, *wnt1, eng2a*, and *pax2a* expression are initiated but are reduced as the MHB forms (Reifers et al., 1998). Timing of MHB development appears important and is complicated by the multiple Wnt genes expressed in the MHD. We have examined how timing of Wnt signaling and the overlapping expression patterns of multiple Wnt genes in the MHD affect its formation.

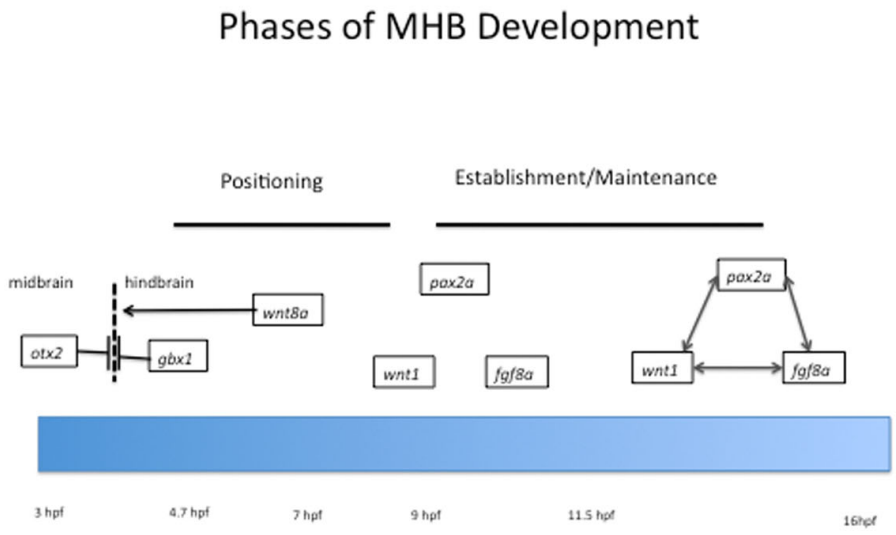

Fig. 1. The phases of MHB development. The three phase of MHB formation are depicted. In the positioning phase, *wnt8a* regulates *otx2* and *gbx2* to position the MHB. In the establishment phase, *pax2a*, *wnt1*, and *fgf8a*, are activated and specify MHB cell fates. In the maintenance phase, *pax2a*, *fgf8a*, and *wnt1* form a regulatory loop, which maintains MHB development.

What Role Does Timing Play in Regulating Multiple Wnt Genes During MHD Development

We examine the role of Wnt signaling in the spatial and temporal patterning of the MHD. Previous studies focused on gain-of-function or loss-of-function experiments to examine regulation of MHD formation. *wnt8a* is activated at 3 hpf, before the MHB is positioned by *otx2* and *gbx1*. *wnt1, wnt10b,* and *wnt3a* are initiated at 8-9 hpf, and *wnt3* is activated at 10 hpf, during the establishment phase of MHB formation (Lekven et al, 2001; Lekven et al 2002; Clements et al 2009). Wnt signaling has been shown to play roles in both neural posteriorization and MHB development (Nordstrom et al., 2002;

Lekven et al., 2002; Buckles et al., 2004). We have examined if there is a relationship between the process of neural posteriorization and induction and patterning of the MHD.

wnt1, *wnt10b*, *wnt3*, and *wnt3a* have overlapping expression patterns in the MHD in zebrafish. *wnt1*, *wnt3a*, and *wnt10b* are activated earlier than *wnt3* and work in combination during MHB development (Buckles et al. 2004; Clements et al 2009). We have examined if the role of *wnt3* in MHB development is separate from that of *wnt1*, *wnt10b*, and *wnt3a*.

Zebrafish as a Model Organism

We use zebrafish as our model organism. Zebrafish make an excellent specimen to study the genetic interactions regulating MHD patterning and formation. Zebrafish fertilize externally and spawn large clutches of embryos, which develop rapidly. In the first 24 hours of development, the body plan is set up and the brain structures are present (Kimmel et al., 1995). The embryos are surrounded by a clear chorion that allows you to examine what is occurring in the developing embryos. Many tools have been developed which allow for easy genetic manipulation of the embryo (Kimmel et al., 1995). Also, multiple mutant phenotypes have been found through large genetic screens (Dooley and Zon, 2000). These mutants have allowed for examination of many developmental processes including organogenesis (Dooley and Zon, 2000).

CHAPTER II

VERTEBRATE NERVOUS SYSTEM POSTERIORIZATION: GRADING THE FUNCTION OF WNT SIGNALING*

Introduction

The vertebrate CNS is subdivided along its anteroior posterior (A/P) length into four broad morphologically distinct domains, forebrain, midbrain, hindbrain, and spinal cord, that arise from the embryonic neural plate. CNS regionalization begins during gastrulation when A/P positional information is established and interpreted by cells in the neural plate into the formation of gross divisions that are each further patterned by localized signaling centers. Patterning within each subdivision produces a characteristic distribution of neuronal cell types with specific connectivity within and between divisions that are essential for nervous system function. Thus, the formation of normal circuitry in the CNS depends critically upon the initial mechanism that establishes and interprets A/P positional information in the neural plate. Consequently, the mechanism of early A/P regionalization in the vertebrate CNS has been a subject of intense study. Because recent results show that altering the strength of signaling centers that pattern

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brain subdivisions may be one mechanism behind the evolution of different brain morphologies (Sylvester et al., 2010; Sylvester et al., 2011), understanding how positional information is established and interpreted during neural A/P patterning is essential for understanding brain development and its evolution. Neural A/P patterning has traditionally been reviewed in the context of neural induction, reflecting historical approaches that viewed neural induction and A/P patterning as outcomes of the same mechanistic process (De Robertis and Kuroda, 2004). However, significant progress has been made in determining the molecular nature of neural A/P patterning and it is thus worthwhile to examine hypotheses of A/P patterning mechanisms that have been developed as a result. The purpose of this review is to focus on studies that have examined the establishment of A/P polarity in the early neural plate and the role of Wnt signaling in establishing A/P positional information in the neural axis. We provide a critical analysis of current mechanistic models stemming from these studies, and propose alternative models that may reconcile recently published reports.

Neural Induction and A/P patterning: the Activation-Transformation Model

Neural induction and neural A/P patterning were initially viewed as a single process, as, for example, Otto Mangold discovered the ability of the Spemann-Mangold Organizer to induce neural tissue characteristic of different A/P positions (Mangold, 1933). That is, grafts of early or late organizers into the blastocoel of a host induced secondary axes with neural tissue of anterior or posterior character, respectively. These results were interpreted to be evidence of region-specific neural inducers. These early hypotheses proposing multiple region-specific neural inducers were eventually discarded

in favor of models proposing that neural induction and A/P patterning were distinct processes. Nieuwkoop elaborated in 1952 what has become a highly influential two-signal model for neural patterning based on experiments in amphibians in which undifferentiated ectoderm "flaps" from early gastrula embryos were transplanted to different positions in the A/P neuraxis of host embryos ranging from gastrula to neurula stages (Nieuwkoop, 1952). From his analysis of the differentiation of the transplanted tissue, Nieuwkoop proposed that neural induction occurs in two phases (the "Activation-Transformation" model): in the first phase a signal induces anterior neural tissue (prosencephalic structures), while in the second phase a signal transforms posterior neural plate into neural tissue of caudal fates (Fig. 2A). Shortly thereafter, Saxén and Toivonen proposed a model whereby a forebrain inducing activity and a mesoderm inducing activity intersect to generate A/P specificity of neural induction (Toivonen and Saxén, 1955). Importantly, Saxén and Toivonen hypothesized that the two interacting inducing activities existed as chemical gradients, based on experiments in which greater amounts of mesoderm inducing tissue in grafts and explant assays led to the formation of progressively posterior neural tissue (Fig. 2B) (Toivonen and Saxen, 1968). While these two hypotheses were not mutually exclusive, Nieuwkoop appeared to favor the idea that induction of posterior neural tissue was the result of the action of more posterior archenteron roof interacting with differentially competent ectoderm rather than being the result of the interpretation of a chemical gradient across the neural plate (Nieuwkoop, 1999). Nieuwkoop's activation-transformation model has received strong experimental support, while the idea that morphogen gradients may be responsible for generating A/P

positional values in the neural plate has received considerable attention. In fact, the eventual identification of endogenous molecules capable of posteriorizing neural tissue has led to currently accepted models that have blended the concept of morphogen gradients with Nieuwkoop's activation-transformation hypothesis.

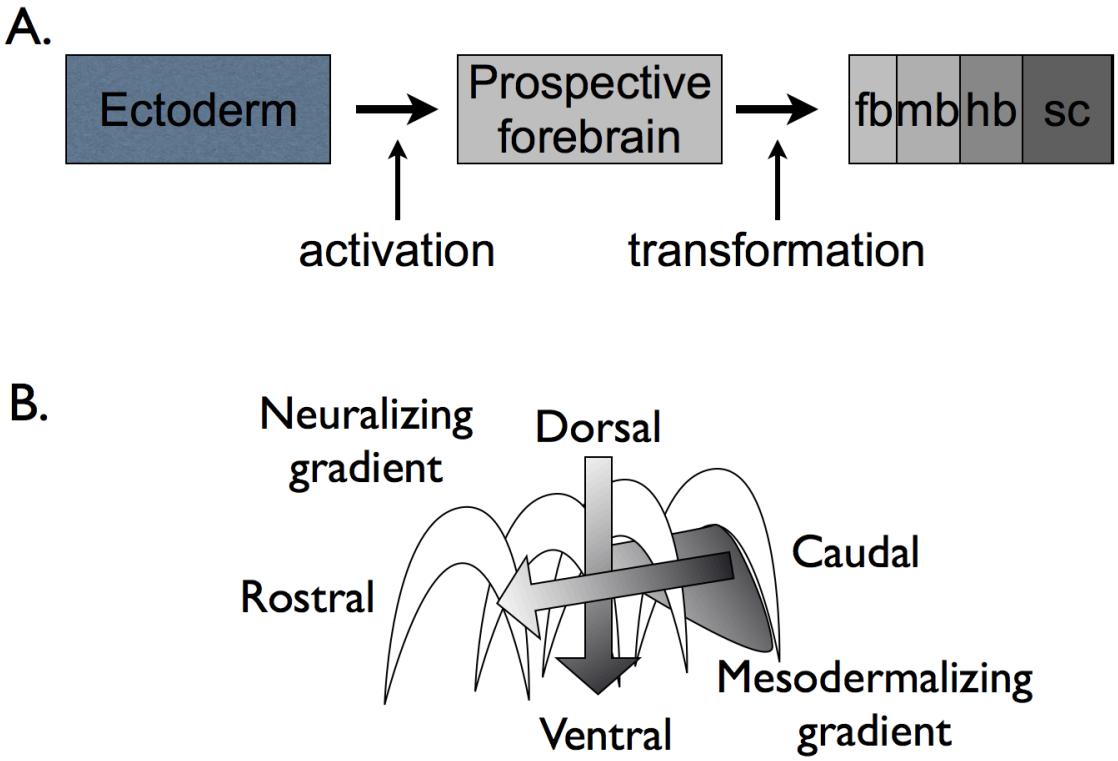

Figure 2. Historical models for early neural plate patterning. A) The Activation-Transformation model of Nieuwkoop. Activation induces neural ectoderm of forebrain character. Subsequent transformation establishes midbrain, hindbrain and spinal cord domains within the neurectoderm. B) The double gradient model of Saxén and Toivonen. A neuralizing gradient establishes neural plate with dorsal-ventral polarity. A subsequent mesodermalizing gradient polarizes the neurectoderm to induce posterior domains. The gradient is highest posteriorly where mesodermal cells are most abundant. (adapted from (Saxen, 2001)).

Identification of posteriorizing molecules and support for the activation-transformation model

The first identification of endogenous candidate posteriorizing agents did not occur until the late 1980s, when Durston et al. showed that treatment of *Xenopus* embryos with retinoic acid (RA) induced microcephaly (Durston et al., 1989). Treated embryos lacked forebrain and midbrain due to a rostral shift of more caudal neural fates, suggesting RA could divert anterior neural tissue to a more posterior fate. Subsequently, studies in the mid to late 90s suggested Fgfs and Wnts as possible posteriorizing signal candidates. Cox and Hemmati-Brivanlou showed that Fgf could mimic the ability of posterior mesoderm to induce the hindbrain marker *egr2* (previously called *krox-20*) and the spinal cord marker *hoxa9* (previously called *Xlhbox6*) in forebrain or neuralized ectoderm explants (Cox and Hemmati-Brivanlou, 1995). McGrew et al. showed that neuralized Xenopus animal caps could be induced by Xwnt3a or the Wnt effector b-catenin to express *en-2*, *egr2* and *hoxa9* (McGrew et al., 1995), and conversely that blocking Wnt signaling by overexpressing DN-Xwnt-8 suppressed *egr2* and *HoxB9* (McGrew et al., 1997).

These and other analyses have shown that Wnt, Fgf, and RA are expressed in the appropriate spatio-temporal pattern to be the endogenous posteriorizing agents; yet, Wnt, Fgf, and RA clearly do not perform equivalent functions. Evidence suggests RA is instructive for posterior identity, and not for determining the relative placement of subdomains in the A/P axis (Kudoh et al., 2002). And while Wnt and Fgf are both sufficient to induce hindbrain and spinal cord gene expression, studies have

demonstrated that Fgf is not sufficient to alter A/P gene expression patterns associated with posteriorization (McGrew et al., 1997; Woo and Fraser, 1998; Nordstrom et al., 2002). For instance, Woo and Fraser demonstrated that Fgf-coated beads could not mimic the ability of the zebrafish embryonic margin, which expresses Wnt and Fgf ligands as well as the RA synthesis enzyme *raldh2*, to induce *egr2* expression in prospective forebrain (Woo and Fraser, 1997). In contrast, several studies have revealed an essential role for Wnt signaling in A/P patterning through direct action on the posterior neural plate (Kiecker and Niehrs, 2001; Nordstrom et al., 2002; Rhinn et al., 2005).

Posteriorization and the Wnt gradient model

Wnts are vertebrate orthologs of Drosophila Wingless, which had been shown to function as a morphogen to induce differential cell fates in the wing disc (Neumann and Cohen, 1996; Zecca et al., 1996). Thus, the identification that Wnt genes were capable of affecting A/P neural patterning eventually led to the tempting speculation that these could function as morphogens in vertebrate neural A/P patterning (Kiecker and Niehrs, 2001). Two central studies elaborated this idea and support the hypothesis that Wnts are expressed in a gradient in the vertebrate neural plate and function as morphogens to differentially induce posterior neural fates. Here we will critically analyze the evidence for and against the Wnt gradient hypothesis.

Kieker and Niehrs in 2001 provided strong evidence that differential Wnt signaling determines neural plate A/P patterning (Kiecker and Niehrs, 2001). Using Xenopus as a model, they assayed for the existence of a dose response to Wnt across the

A/P axis using a combination of in vivo and in vitro techniques. Xenopus animal cap explants were soaked in recombinant XWnt8 proteins of different concentrations. In the absence of exogenous XWnt8 protein, animal cap cells neuralized by dissociation express *BF1*, a forebrain marker, and *otx2*, a forebrain and midbrain primordium marker. When a low XWnt8 concentration was applied, the neuralized animal cap cells did not express *BF1* but expressed *otx2* and *en-2*. When a higher XWnt8 concentration was applied, the neuralized cells expressed *egr2*. In embryos, ectopic expression of Wnts or antagonism of endogenous Wnt signaling resulted in a graded shift in neural markers toward the anterior (with overexpression) or posterior (with antagonism), consistent with the finding that higher levels of Wnt signaling are associated with posterior neural fates. This predicts that the posterior nervous system should experience higher Wnt signaling levels. To measure spatial levels of Wnt signaling in vivo, they utilized two bioassays of Wnt signaling: the TOP-Flash reporter, which is transcriptionally activated by Wnt signaling, and nuclear localization of b-catenin, a downstream output of signaling. They found that late gastrula stage embryos showed high levels of Top Flash activation and nuclear b-catenin at the most posterior regions of the neural plate, moderate levels in the middle region corresponding to the presumptive hindbrain and midbrain, and low levels in the anterior region, corresponding to prospective forebrain (Kiecker and Niehrs, 2001).

The following year, Nordstrom et al. presented data supporting the idea that Wnt signaling levels pattern the chick A/P neural axis (Nordstrom et al., 2002). In one approach, the authors grew chick neural plate explants in medium containing Wnt3A and

Fgf8 proteins of different concentrations, assayed A/P restricted gene expression and observed a graded response of the neural plate to Wnt3A with low/no Wnt3A inducing forebrain fate (Otx2+/Pax6+), moderate Wnt3A inducing midbrain fate (Otx2+/En1+) and high levels of Wnt3A inducing hindbrain fate (Gbx2+, Krox20+) (Nordstrom et al., 2002). Together, the studies of Kiecker and Niehrs and Nordstrom et al. provide compelling evidence that progressively higher levels of Wnt signaling experienced by the posterior neural plate are responsible for the acquisition of specific posterior neural fates. Further, Kiecker and Niehrs proposed the intriguing parallel between axis patterning in the vertebrate axes and the Drosophila wing imaginal disc, wherein orthogonal gradients of Wnt and BMP signaling may constitute an evolutionarily conserved module to pattern intersecting axes (Kiecker and Niehrs, 2001).

Studies in zebrafish paint a supportive picture. In zebrafish, neural A/P polarization depends upon signaling from the embryonic margin (Woo and Fraser, 1997), where *wnt8a* expression in ventrolateral mesendoderm progenitors is required for posteriorization (Erter et al., 2001; Lekven et al., 2001). Ectopic *wnt8a* expression in the dorsal mesoderm (the shield, i.e. the teleost organizer) suppresses forebrain specification, demonstrating that repression of *wnt8a* in the organizer is essential for normal establishment of A/P patterning (Fekany-Lee et al., 2000; Seiliez et al., 2006). In support of a level-dependent readout of Wnt8a signaling, injections of *wnt8a* mRNA into single cell zebrafish embryos induces the anterior hindbrain marker *gbx1* and represses *otx2* in a dose-dependent manner (Rhinn et al., 2005).

These and other papers (e.g.,Bang et al., 1999; Dorsky et al., 2003) thus elaborate the Wnt gradient model for neural A/P patterning: that Wnts are secreted by nonaxial mesoderm progenitors at the posterior neural plate and diffuse into the neural plate to create a linear gradient with highest levels in the posterior and lowest anterior; the gradient induces a dose-dependent response within the neural plate that leads to the elaboration of gene expression domains to establish the forebrain, midbrain and hindbrain primordia (Fig. 3).

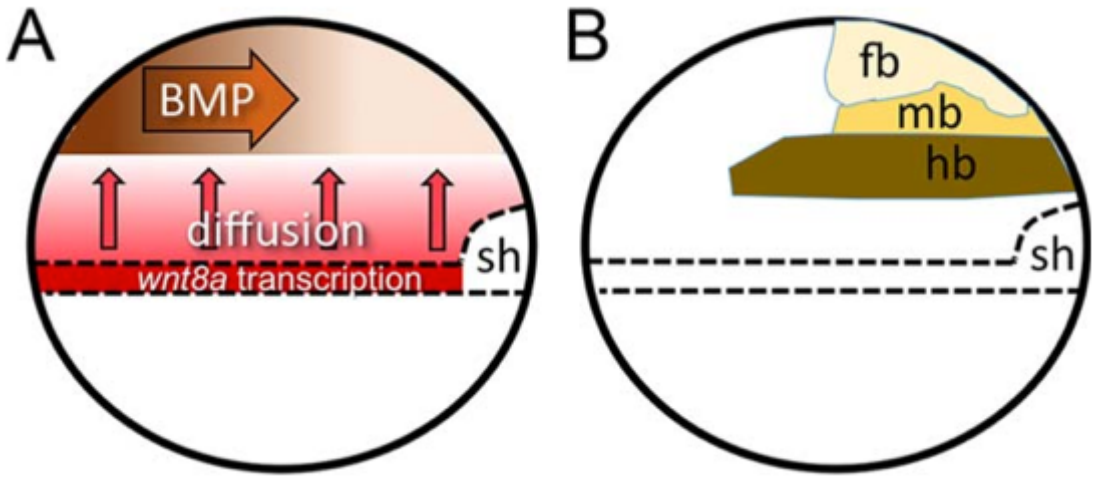

Figure 3. Summary of the Wnt gradient model of neural posteriorization, schematized for a zebrafish early gastrula embryo. Diagrams schematize lateral views of embryos at 6 hours post fertilization (shield stage), dorsal to the right. A) BMP antagonists expressed from the dorsal organizer (in zebrafish, the shield, sh) in the embryo create a gradient of activity that results in neural induction. *wnt8a* transcripts are produced in mesendoderm progenitors at the blastoderm edge (horizontal red line). Protein expressed from these cells diffuses anteriorly (red vertical arrows) to create a Wnt8a protein gradient (pink shading) that induces posterior domains within the neural plate. B) The result of the posteriorizing gradient is the specification of posterior neural domains that have been outlined in fate mapping experiments (see Woo and Fraser, 1995). Fb, mb, hb: forebrain, midbrain and hindbrain primordia.

While the gradient model fits well with many reports, key elements remain to be tested. The inability to assay Wnt protein distribution has made direct measurement of the hypothesized gradient difficult, and recent work in Drosophila showing that membrane tethered Wg is able to pattern the wing disc properly raises the possibility that diffusion through the extracellular space may not be an essential element to Wnt function (Alexandre et al., 2014). The recent finding that vertebrate Shh is transported along long filopodial extensions within the chick limb bud (Sanders et al., 2013) raises the possibility that morphogen transport via cellular extensions may be a general phenomenon, and recent reports suggest this may apply to Wnt8a as well (Hagemann et al., 2014; Luz et al., 2014). Recent evidence has also been obtained which suggests that Wnts can be transported along migrating neural crest cells to mimic a concentration gradient (Serralbo and Marcelle, 2014). Whether these different transport mechanisms are responsible for delivering Wnt ligands to the neurectoderm should be investigated as a necessary step in understanding how graded Wnt signaling is established. Whether through diffusion or via deposition, the shape of the putative Wnt gradient is unknown. In vivo Wnt bioassays, while informative, do not offer the spatial or temporal precision required to determine the shape of the gradient. This leaves the gradient shape open for interpretation with several different possibilities consistent with published data (Fig. 4). New technologies, such as Crispr/Cas mediated chromosome engineering, have opened up new technological possibilities in vertebrate model systems for altering Wnt pathway genes in ways that may help elucidate the nature of the hypothesized gradient (Shin et al., 2014). Therefore, to fully understand this phenomenon, more research must be

performed to determine the nature of the gradient, its shape and how Wnt proteins are delivered across the neural plate.

A relatively unstudied but major aspect of the Wnt gradient hypothesis is timing (Gerhart, 1999). Nieuwkoop theorized that A/P qualities of neural posteriorization may involve the timing of neural plate contact with inducing tissues and also differential ectodermal competence to respond to inducing signals (Nieuwkoop, 1952). As the paraxial mesoderm has been identified as the source of posteriorizing signals, the Wnt gradient hypothesis must be aligned within the larger context of the complex cellular movements of gastrulation when the pattern of the neural plate is being established. For instance, in the developing zebrafish embryo, Wnt8a is produced by mesendoderm progenitors at the blastoderm margin (Kelly et al., 1995; Lekven et al., 2001). As gastrulation proceeds, the margin moves vegetally during the process of epiboly as cell proliferation, convergence and extension grow the neural plate and condense it at the dorsal midline (Solnica-Krezel and Cooper, 2002). Accordingly, the position of forebrain, midbrain and hindbrain progenitors relative to the embryonic margin is highly dynamic during gastrulation (Woo and Fraser, 1995). Cell migration could also be an important factor in shaping the neural plate response to the Wnt gradient by altering the relationship of Wnt ligands and extracellular antagonists. Indeed, the Wnt/b-catenin antagonist Dkk1 was shown to interact with both the Wnt co-receptor LRP5/6 and the glypican 4/6 homolog Knypek to regulate gastrulation movements (Caneparo et al., 2007). Thus, cell migration could have an important role in controlling the time during which cells experience a Wnt signal of a certain threshold (Fig. 4).

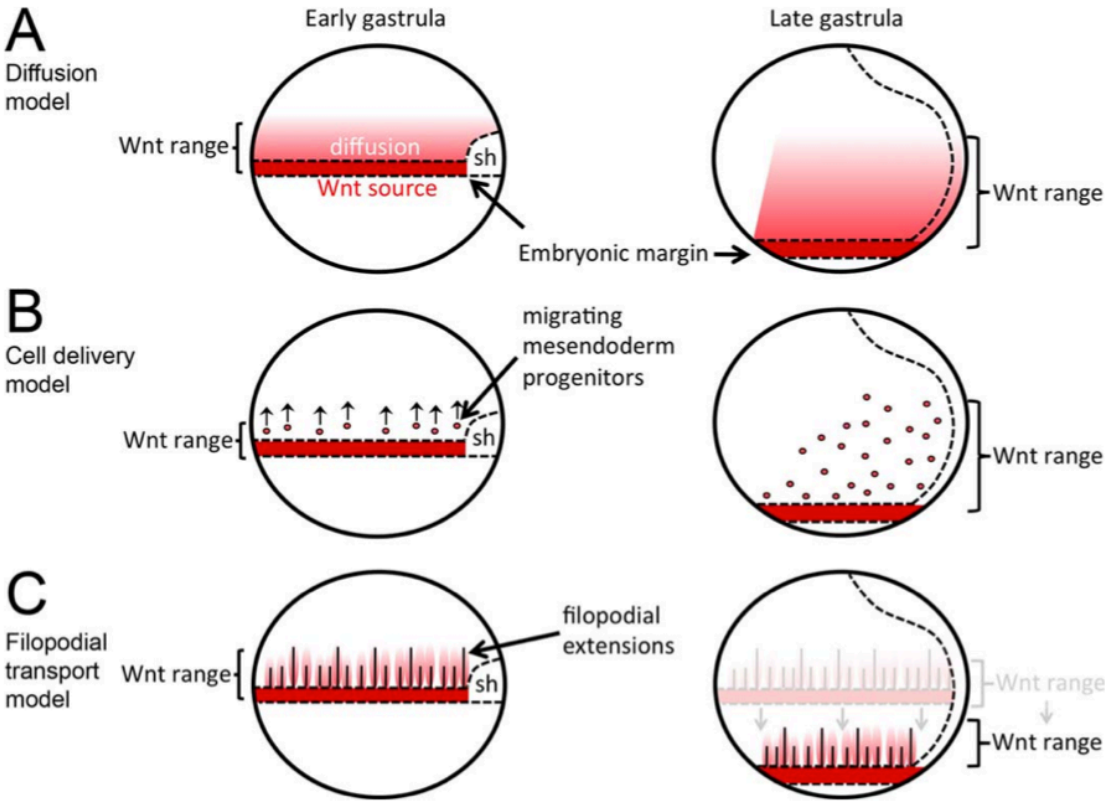

Figure 4. Potential models for Wnt8a delivery across the neural ectoderm schematized for gastrulating zebrafish embryos. All diagrams represent embryos oriented with anterior up, dorsal to right. A) Secretion and diffusion model. Left: Mesendoderm progenitors at the embryonic margin transcribe *wnt8a* (dark red), then secrete protein that diffuses away from the margin to generate a concentration gradient that declines anteriorly (bracket). Right: As gastrulation proceeds, the diffusion gradient spreads to span the posterior neural plate (brackets). B) Migrating cell delivery model. Left: Migrating mesendoderm cells (red circles) express Wnt8a on their surface, and their migration away from the margin (arrows) determines the Wnt8a signaling range. Right: As gastrulation proceeds, Wnt8a signaling range is determined by distribution of migrating cells. C) Filopodial transport model. Left: Wnt8a produced in marginal mesendoderm progenitors is transported along filopodial extensions away from the margin (black vertical lines). The signaling range of Wnt8a is then limited by the dynamics of filopodial extension. Right: As gastrulation proceeds and the embryonic margin migrates toward the vegetal pole, the Wnt8a signaling range remains a consistent distance from the margin.

Downstream of Wnt signaling: a gradient of de-repression?

Regardless of whether posteriorizing Wnts function through a diffusion gradient or some other mechanism, the next question is how graded Wnt signaling is translated by responding cells in the neural plate to establish A/P- restricted fate zones. The mechanism of the neural plate response to Wnt signaling is not well established. In the canonical Wnt pathway, active signaling causes the stabilization of cytoplasmic β-catenin protein, which eventually translocates to the nucleus to act as a transcriptional co-activator of Lef/Tcf family transcription factors (Hoppler and Kavanagh, 2007). In the absence of Wnt ligand, some Lef/Tcf transcription factors at the promoter of Wnt responsive genes interact with Groucho transcriptional repressors (Cadigan and Waterman, 2012). Thus, Wnt signaling can act through two transcriptional mechanisms: de-repression or activation. In the de-repression mechanism, active Wnt signaling results in indirect activation of target genes by removing repression imposed on these genes by Tcf-Groucho complexes. In the activation mechanism, Wnt signaling results in the direct transcriptional activation of target genes through the recruitment of CBP/p300 or other activating proteins (Cadigan and Waterman, 2012). The determining factor between de-repression and activation appears to be the identity of the Lef/Tcf protein involved (Cadigan and Waterman, 2012). This is relevant to discussions of neural posteriorization, as the major function of Wnt signaling in posteriorization may be to counter Tcf3-dependent transcriptional repression (Kim et al., 2000; Dorsky et al., 2003). For example, zebrafish *headless* (*hdl*) mutants have a mutation in *tcf7l1a* (formerly called *tcf3*) that results in anterior brain deficiencies and overall caudalization

of the brain (Kim et al., 2000). *hdl* mutant embryos can be rescued by a dominant-negative form of Tcf3 that lacks the β-catenin binding domain but retains repressor activity. This suggests that Tcf3 represses posterior gene expression to pattern anterior neurectoderm. Conversely, loss of posteriorizing Wnt function results in caudal expansion of forebrain gene expression such as *pax6* (Lekven et al., 2001; Dorsky et al., 2003). In zebrafish, simultaneous inhibition of *tcf3* and posteriorizing Wnt activity results in relatively normal A/P patterns of gene expression in the neural ectoderm at the end of gastrulation, suggesting that de-repression of Tcf3 activity is a primary function for Wnt in neural posteriorization (Dorsky et al., 2003; Andoniadou et al., 2011). If de-repression of Tcf3 is the primary role of posteriorizing Wnt signaling, it would argue that the induction of posterior fate is mediated through some other signaling pathway, prime candidates being Fgf and RA signaling.

Resolving the interdependence of Wnt, Fgf and RA signaling during neural posteriorization

Wnt signaling clearly plays a central role in posteriorization, but the fact that Fgf and RA signaling are also capable of posteriorizing neural tissue indicates that these must play some role in relation to the Wnt gradient. Nordstrom et al. reported that Fgf signaling was required to observe dose-dependent posterior neural fate specification by Wnt ligands in chick neural plate explants, but suggested that Fgf signaling functioned permissively to allow the response to Wnt (Nordstrom et al., 2002). Other studies have suggested interdependence of Wnt and Fgf signaling in mediating posteriorization. For example, McGrew et al. (McGrew et al., 1997) showed that Xwnt-3a mediated

suppression of *otx2* in neuralized Xenopus animal caps requires Fgf signaling, but Xwnt-3a induction of *en-2* and *egr2* did not. However, Fgf treatment partially suppressed *otx2* and activated *en-2* and *egr2*, but this depended on Wnt signaling. In contrast, Domingos et al. suggested that Wnt signaling posteriorizes the neural plate indirectly in a process that depends upon Fgf signaling (Domingos et al., 2001). These authors used a method to cell-autonomously activate the Wnt pathway with temporal control, based on expressing a fusion protein comprising the Lef1 DNA binding domain, the b-catenin C-terminal transactivation domain and the ligand binding domain of the human glucocorticoid receptor to render it inducible by dexamethasone. They showed that activation of this fusion protein in animal caps can suppress the anterior marker *BF-1* and activate *en-2* and *Hoxb9*. Importantly, as revealed by in situ hybridization, the induction of *en-2* and *Hoxb9* occurs in cells outside of those in which Wnt signaling was activated, indicating that *en-2* and *Hoxb9* induction in this assay occurred cell non-autonomously. The induction of *en-2* and *Hoxb9* is suppressed in animal caps with compromised Fgf signaling, leading to the hypothesis that Wnt signaling indirectly controls A/P patterning via regulation of Fgf (Domingos et al., 2001). In intact whole embryos, expression of the cell-autonomous activator of the Wnt pathway in the neural plate resulted in a global shift of *en-2* and *egr2* expression domains toward the anterior embryo, an observation that also fits best with the de-repression hypothesis since overall changes in neural plate patterning observed did not strictly correlate with the cells in which Wnt signaling was activated. However, the suggestion that Wnt signaling acts indirectly to control A/P patterning contradicts evidence in zebrafish, Xenopus, and

chick that Wnt signaling acts directly on the neural plate to control polarization (Kiecker and Niehrs, 2001; Nordstrom et al., 2002; Rhinn et al., 2005). This discrepancy may reflect the nature of the cell autonomous Wnt pathway activator utilized by Domingos et al.: if the normal Wnt response reflects relief of Tcf-dependent repression mediated through the Groucho co-repressor, then transcriptional activation mediated through the fusion protein may not accurately reflect the normal process occurring in vivo.

Importantly, these studies suggest that suppression of forebrain/midbrain gene expression and activation of midbrain/hindbrain gene expression reflects alternative modes of integrating posteriorizing signals. Kudoh et al. explored Wnt-Fgf-RA integration in neural posteriorization in zebrafish, and suggested a model in which Fgf and Wnt signaling function initially to suppress expression of *otx2* and *cyp26*, which then allows subsequent production of RA to induce *hoxb1b* (and other posterior neural genes) in the posterior neural plate (Kudoh et al., 2002).

Conclusions

Significant progress has been made in understanding the molecular control of early neural A/P patterning, but many questions remain. Various approaches in different model organisms generally support modern iterations of Nieuwkoop's activation-transformation model, and the important role for graded Wnt signaling in the process, yet major discrepancies exist in data pertaining to the relative functions of Wnt, Fgf and RA signaling. One limitation of many studies is the relatively small number of molecular markers used for analysis, which define few gene expression domains and thus offer a relatively low level of cell lineage resolution. A consensus has emerged that

posteriorization signals create a gradient of information that establishes broad A/P-restricted domains, which set the position of secondary organizers, such as the midbrain-hindbrain boundary, that then further refine patterning within each gross domain. The use of methods with relatively low resolution of cell lineages may produce an unclear picture of the timing of sub-domain patterning and specification, and thus the dynamics of neural patterning. For example, a zebrafish study of forebrain patterning that used approaches with high spatial and temporal resolution of cell lineages revealed that the prethalamus is determined during gastrulation and patterns adjacent diencephalic fates (Staudt and Houart, 2007). Thus, low-resolution methods leave unanswered several important questions such as how transitions between domains are established and how fate specification intersects with morphogenetic mechanisms. Clearly, there is much work yet to be done.

CHAPTER III

THE FUNCTIONAL ROLE OF WNT SIGNALING IN THE TEMPORAL AND SPATIAL PATTERNING OF THE MHD IN ZEBRAFISH

Introduction

The zebrafish midbrain hindbrain domain (MHD) consists of the midbrain, midbrain hindbrain boundary (MHB), and anterior hindbrain, and corresponds to the mes-r1 region in mouse. This domain is derived from patterning of the anterior neural tube, which is dependent on Wnt/b-catenin signaling through a mechanism that is not understood. We assess the potential role of Wnt signal timing in patterning the MHD. MHD formation and patterning is invariably intertwined with the midbrain-hindbrain boundary, which is proposed to be specified and patterned in three phases: positioning, establishment, and maintenance.

The zebrafish MHB is first positioned at the interface of *otx2* expression in presumptive midbrain and *gbx1* expression in the presumptive hindbrain at 6.5 hours post fertilization (hpf) during the positioning phase (Rhinn et al., 2005). Subsequent to positioning, the development of the MHB is next established by the activation of *pax2a*, *wnt1*, and *fgf8a* at 8 hpf which form a genetic regulatory network (GRN) (Rabile and Brand, 2004). During maintenance, the MHB GRN is maintained by the *pax2a-wnt1-fgf8* regulatory loop beginning at approximately 11 hpf (Rabile and Brand, 2004).

The Wnt pathway is involved in many different processes including cell proliferation, apoptosis, and cell fate specification. *wnt1* is expressed in the dorsal

midbrain and MHB, while *wnt3a* is expressed in the dorsal neural tube, midbrain, and hindbrain (Ikeya et al., 1997; Roelink and Nusse, 1991). Studies show both these genes have a functional role in patterning the MHD. In mouse embryos, deletion of *wnt1* causes a loss of the midbrain, MHB and anterior hindbrain, while deletion of *wnt1* and *wnt3a* causes a reduction in hindbrain (Danielian and McMahon, 1996; Ikeya, et al., 1997). In zebrafish, *wnt10b* expression overlaps with *wnt1* and deletion of both these genes reduces the MHB constriction (Lekven et al., 2002), and further reduction of *wnt3a* in *wnt1/wnt10b* mutants results in MHB absence (Buckles et al., 2004).

The interaction of genes regulating the formation of the MHB is complex. Studies in zebrafish show that *pax2a* and *eng2a* expression in the ventral MHB is dependent on *wnt1;wnt10b* but expression in the dorsal MHB is not. However, *fgf8a* expression in the MHB is similar to wild type in *wnt1;wnt10b* loss-of-function embryos (Lekven et al, 2002). Loss of *wnt1*, *wnt10b* and *wnt3a* causes a complete loss of *pax2a* and *eng2a* at 24 hpf (Buckles et al, 2004). While studies of Wnt mutants have revealed functional requirements in MHB development, studies have overlooked how timing of Wnt signaling affects MHB formation. We hypothesize distinct functional windows in Wnt signaling roles in MHD specification and maintenance.

We examined the effect of Wnt signal timing on the spatial patterning of the MHD by inhibiting Wnt signaling during developmental intervals and assaying molecular markers of the MHD. We then examined how the timing of Wnt signaling affects regulation of the MHB GRN. We found that Wnt signaling is required for

posteriorization of the neural plate during gastrulation by promoting midbrain and hindbrain development while repressing forebrain expansion.

Methods and Materials

Zebrafish care

Zebrafish were maintained as described (Westerfield, 2000). An AB-TL hybrid line serves as our wild-type stock. The Texas A&M Institutional Animal Care and Use Committee approved vertebrate animal procedures. The HSdkkGFP/+ line was obtained from Dr. Randall Moon (University of Washington) (Stoick-Cooper et al., 2007).

Heat shocks

HSdkkGFP/+ were crossed to wild-type adult fish. Embryos were collected within 15 minutes of fish spawning. Zebrafish embryos are normally kept at 29°. To perform heat shocks, embryos were placed in PCR tubes in groups of 10 per tube. Embryos were incubated at 37 ° for one hour, then returned to 29 °. Embryos were heat shocked at 3. 4.3, 4.7, 7, 14, and 16 hpf. At 24 hpf, embryos were examined for morphological brain defects. Embryos were fixed at 11.5, 14. 16, and 24 hpf using 4% paraformaldhyde overnight at 4°. Unless otherwise indicated, hs:*dkk1*/+ embryos were unambiguously identified after heat shock by morphological criteria and represented ~50% of offspring of the HSdkkGFP/+ X +/+ crosses.

In situ hybridizations

Our protocol is a modification of Oxtoby and Jowett (1993). Fixed embryos were dechorionated in PTW (1X PBS, 0.1% Tween-20). Embryos were stored at -20°C in methanol. Embryos were rehydrated by one wash of 50% methanol/50% PTW for 5

minutes and then 2-3 washes of PTW for 5 minutes. Embryos were treated with 1 ul 10mg/ml proteinase K in 1 ml PTW. Embryos at 11.5, 14, 16 hpf were treated for 5-10 minutes, while 24 hpf embryos were treated for 20-25 minutes. Embryos were washed 2X with 100 mg/ml glycine in 50 ml PTW and then refixed in 4% paraformaldhyde for 20 minutes. Embryo were washed 2X with PTW for 5 minutes. Embryos are placed in a hybridization buffer (50% formamide, 5X SSC, 0.1% Tween-20; 50ug/ml heparin,500 ug/ml tRNA and 5% dexatran sulfate) at 67° for an hour. The digoxigenin labeled RNA probe (0.5 ng/ul-1ng/ul) is placed on the embryos over night at 67° C.

The next day embryos are washed 2X at 67° for 30 minutes with 2X SSC, 50% formamide, and 0.1% Tween-20. Embryos are then washed once for 15 minutes at 67° with 2X SSC, and 0.1% Tween-20 and then 2X at 67° for 30 minutes with 0.2X SSC, and 0.1% Tween-20. Embryos are washed 2X with PTW for 5 minutes at room temperature and placed in blocking solution (1.5 ml FBS in PTW for 15 ml total). At room temperature embryo are incubated for either 2 hours at room temperature or overnight at 4° in antibody (1:2000 for 2 hours, 1:5000 overnight).

Embryos are washed 4X in PTW for 15 minutes each. Next embryos are placed in a staining buffer (100mM Tris pH 9.5; 50nM MgCl2, 100nM NaCl; 0.1% Tween-20) Embryos are placed in a color reaction solution of 4.5 ul/ml NBT and 3.5 ul/ml BCIP in staining buffer and stopped after reaction using methanol. Embryos then are placed in a graded glycerol series and kept in 70% glycerol.

Our double in situ hybridization protocol is modified from Jowett (2001). When the probe is placed on the embryos, it is a mixture of the digoxigenin labeled RNA probe

(0.5 ng/ul-1ng/ul) and the fluorescein labeled RNA probe (1-2 ng/ul). The NBT/BCIP color reaction is stopped with 4% paraformaldehyde/staining buffer for 2 hours at room temperature or 4º overnight. All washes are performed at room temperature. Embryos were rinsed rinse 2X with staining buffer. Embryos are washed with 1X PBS for 10 min and then 2X in blocking solution for 15 minutes. Embryos are placed in blocking solution for an hour and then the antibody (1:500) for 2 hours. Embryo are then washed 5X for 12 minutes in blocking solution. Embryos are washed 2X in staining buffer for 5 minutes. Embryos are placed in a color reaction solution of Fast Red dissolved in 2 ml 0.1 M Tris pH 8.2 until reaction is stopped by a 15 minute wash with staining buffer and then with 4% paraformaldehyde/staining buffer for 2 hours. Embryos are kept in glycerol.

Probes used were *pax2a, eng2a, fgf8a,* and *wnt1,* which are expressed in the MHD (Krauss et al., 1991; Molven et al., 1991; Haffter et al., 1996; Fjose et al., 1988) . *otx2* is expressed in the presumptive midbrain (Li et al., 1994). *egr2a,* which is expressed in the hindbrain and *epha4a*, which is expressed in the forebrain and hindbrain were used (Oxtoby and Jowett, 1993; Xu et al., 1994). *zic1* and *dmxb1a* which are expressed in the forebrain (Grinblat et al., 1998; Kawahara et al., 2002). *hoxb1b* is expressed in the posterior neural plate and *gbx2* is expressed in the anterior hindbrain (Eisen and Weston, 1993; Reim and Brand, 2002) . *sp5a* and *sp51* which are expressed in this MHD were used (Weidinger et al., 2005; Tallafuss et al., 2001).

Imaging

To image, embryos are placed in well plates with 100% glycerol. We used Spot

imaging software to image embryos. Images were processed using Photoshop.

Results

Temporal control of Wnt signaling with the Tg(hsp70l:dkk1b-GFP) transgene

To examine the role of Wnt signal timing in MHD development, we used a transgenic line of zebrafish in which *dkk1b,* a Wnt signaling inhibitor, is under the control of the Hsp70-like promoter (Stoick-Cooper et al., 2007). We crossed the transgenic line with wild-type adults and heat shocked the embryos at specific developmental intervals. Half of the offspring from this cross should be transgenic, and half serve as a wild-type control. Because the duration of Wnt antagonism in embryos from this line had not been previously characterized, we first assayed the Wnt target gene, *axin2*, by in situ hybridization at regular 30 minute intervals after applying a heat shock from 3-4 hpf. *axin2* transcripts are known to be reduced for at least two hours in *hs:dkk1*/+ embryos after a one-hour heat shock (Stoick-Cooper et al., 2007). Consistent with this previous report, *axin2* levels are visibly reduced in approximately half of embryos derived from hs:*dkk1*/+ X +/+ crosses at the end of the one hour heat shock (9/23 embryos with visibly reduced staining), (Fig. 5). By 5 hours post heat shock, *axin2* levels in treated embryos are not visibly different from wild-type (24/24 embryos with similar staining), thus we infer that Wnt signaling is inhibited for a 4-5 hour period after our heat shock regimen.

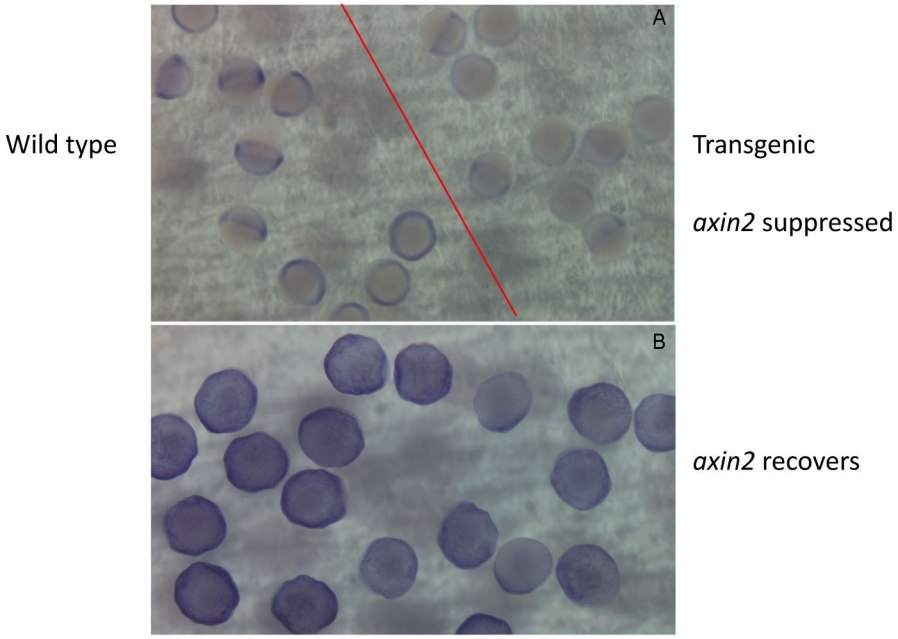

Figure 5. *dkk1b* inhibits Wnt signaling. Top panel: immediately after heat shock, embryos can be sorted into those with reduced staining (right of red line) and those with normal staining (left of red line). We infer these represent hs:dkk1b/+ and +/+, respectively. Bottom panel: 5 hours post heat shock, *axin2* is similar in all embryos.

To address Wnt requirements at different developmental times, we performed a series of heat shock experiments in which heat shock was initiated at progressively later developmental time points. From the *axin2* in situ hybridization, we infer that the window of inhibition extends for a 4-5 hour period after the end of the heat shock.

Positioning phase Wnt signaling disrupts midbrain and hindbrain development, but not MHB formation

Inhibition of Wnt signaling at 3 hpf produces three phenotypic classes of dorsalized embryos in the previously described C1-C5 classification scale (Kishimoto et

al., 1997), which is consistent with the absence of *wnt8a,* the only canonical Wnt gene expressed at this time (Lekven et al., 2001). In the heat shocks performed at 3 hpf, three of the dorsalized classifications were observed: C5, the most severe, with embryos consisting of a head with eyes but no trunk or tail (7/22 transgenic embryos), C4, with embryos consisting of a head with a portion of the trunk but no somites or tail (7/22 transgenic embryos), and C3, the least severe, with embryos having a slightly enlarged head with a smaller MHB constriction, eye, and otic placode (4/22 transgenic; transgenics and non-transgenic siblings were identified unambiguously by morphological criteria, see Materials and Methods) (Fig. 6A,B). When embryos are heat shocked at 4.3 or 4.7 hpf, the forebrain, midbrain, and MHB appear similar to wild type, while the anterior hindbrain appears reduced (4.3 hpf heat shock: 16/16 transgenic embryos with phenotype; 4.7 hpf heat shock: 22/22 transgenic embryos with phenotype) (Fig. 6C, Fig. 7A,B). Therefore, Wnt signaling is required for global anterior-posterior patterning of the neural plate but not MHB development between 3 and 9.5 hpf.

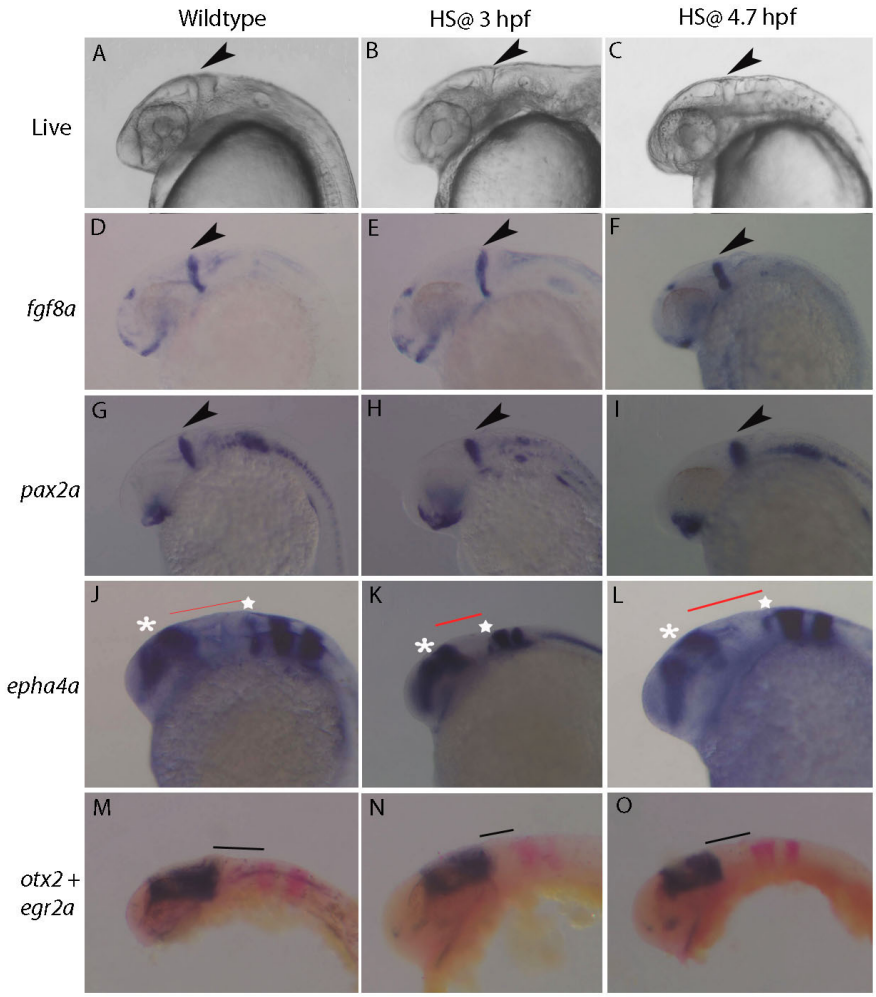

Figure 6. Midbrain and hindbrain, but not MHB formation are disrupted with early stage inhibition of Wnt signaling. Lateral views of heads at 24 hpf. Heat shocked embryos show a deformed midbrain and hindbrain, but the MHB is present (A-C). *fgf8a* and *pax2a* expressed in the MHB are similar to wild type (MHB marked by the black arrows) (D-I). *epha4a* expressed in the forebrain (marked by the white asterisk) appears similar to wild type, while r1 in the anterior hindbrain is disrupted (marked by the white star) (the red line represents MHD) (J-L). *otx2* is expressed in the midbrain (in blue) and appears smaller and *egr2a* in r3 and r5 in the hindbrain (in red) shifts anteriorly (the black line represents the gap in between midbrain and r3) (M-O).

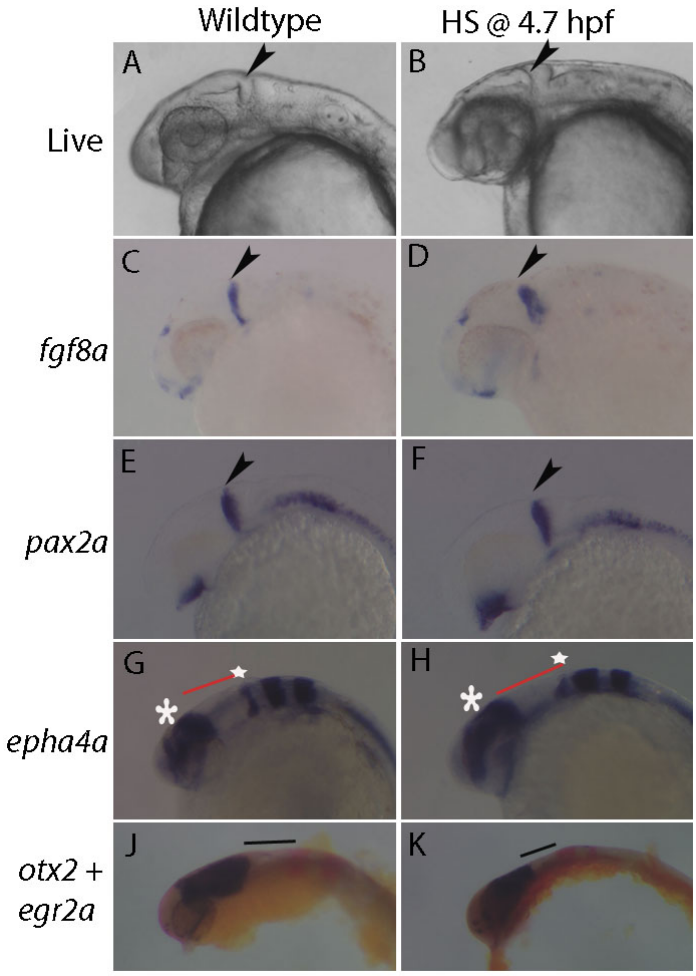

Figure 7. Midbrain and hindbrain, but not MHB formation, are interrupted with 4.3 hpf inhibition of Wnt signaling. 24 hpf lateral views of zebrafish heads. Midbrain and anterior hindbrain, but not MHB appear deformed in live embryos (A-B). *fgf8a* and *pax2a* expressed in MHB are similar to wild type (C-F) (arrows mark the MHB). *epha4a* is expressed in the forebrain appears similar to wild type (marked by the white asterisk) while r1 in the anterior hindbrain is smaller (marked by the white arrow) the red line represents MHD (G-H). *otx2* expressed in the midbrain (in blue) is smaller and *egr2a* expressed in r3 and r5 in the hindbrain shifts to the anterior (in red). Black line represents area between midbrain and r3 (J-K).

To complement our morphological assessment, we assayed forebrain, midbrain, MHB, and hindbrain cell populations by in situ hybridization in positioning-phase Wnt-

inhibited embryos. Consistent with our morphological observations, all domains are present in treated embryos. Forebrain and MHB expression in heat shocked embryos is similar to wild type, as indicated by optic stalk and MHB expression of both *fgf8a* (4.3hpf heat shock: 19/19 transgenic embryos with phenotype; 4.7 hpf heat shock: 13/13 transgenic embryos with phenotype) and *pax2a (*3 hpf heat shock: 9/9 scorable (C3, C4 phenotypes) transgenic embryos with phenotype; 4.3 hpf heat shock: 26/26 transgenic embryos with phenotype; 4.7 hpf heat shock: 16/17 transgenic with phenotype) (Fig. 6D-I, Fig. 7C-F), and forebrain expression of *epha4a* (3hpf heat shock: 3/3 scorable (C3, C4 phenotypes) transgenic embryos with phenotype; 4.3hpf heat shock: 19/19 transgenic embryos with phenotype; 4.7 hpf heat shock 17/17 transgenic embryos with phenotype) (Fig. 6 J-L, Fig. 7 G,H). In contrast, the size of the midbrain appears somewhat diminished, as indicated by the unstained region between diencephalic and rhombomere 1 expression domains of *epha4a* (Fig. 4J-L, Fig. 5G,H) and the midbrain domain of *otx2* (Fig. 6M-O, Fig. 7I,J). Wnt disruption during this early period also results in hindbrain patterning defects, as the distance between the r1-r5 expression domains of *epha4a* appears shortened (Fig. 6J-L, Fig. 7G,H), as does the distance between midbrain *otx2* and *egr2a* expression in r3-r5 (3hpf heat shock: 11/11 transgenic embryos; 4.3hpf heat shock: 18/18 transgenic embryos with phenotype; 4.7hpf heat shock: 17/17 transgenic embryos with phenotype) (Fig. 6M-O, Fig. 7I,J). These data suggest forebrain and MHB formation does not require positioning-phase Wnt signaling. However midbrain and anterior hindbrain development does require Wnt signaling during this time, as it is interrupted, perhaps by an expansion of the eye field and concomitant posterior shift of

forebrain territories.

Establishment and maintenance phase Wnt signaling is necessary for MHD formation

We next examined whether inhibition of Wnt signaling at developmental intervals during and after gastrulation affects MHD patterning. Blocking Wnt signaling at 7 hpf results in embryos with severely disturbed brain morphology, with significantly enlarged eyes, and severely reduced or absent midbrain, MHB, and anterior hindbrain (14/14 transgenic embryos with phenotype) (Fig. 8A,B). Inhibition of Wnt signaling at 14 hpf results in a normal forebrain and midbrain, but the MHB is absent and anterior hindbrain appears slightly larger (17/19 transgenic embryos with phenotype) (Fig. 8C). Blocking Wnt signaling at 16 hpf results in normal appearance of the forebrain, midbrain, MHB and anterior hindbrain (29/29 transgenic embryos with phenotype) (Fig. 8D). Thus, normal brain morphology is dependent upon Wnt signaling between 7 and 16 hpf.

We assayed anteroposterior brain domains by in situ hybridization, and, in contrast to the results of early gastrula heat shocks, significant brain domain losses are caused by late gastrula and early somite stage heat shocks. When heat shocked at 7 hpf, forebrain domains of *fgf8a* (7/10 transgenic embryos with phenotype), *pax2a* (8/8 transgenic embryos with phenotype) and *epha4a* are largely normal (28/28 transgenic embryos with phenotype) (Fig. 8B,F,J,N) with the exception that the eyes and optic stalk are enlarged (e.g., abnormal optic stalk *pax2a*, Fig. 6J). In contrast to forebrain, MHB expression of *fgf8a* and *pax2a* is absent (Fig. 8F,J), and this appears accompanied by the absence of midbrain tissue indicated by the close proximity of the diencephalic and r1 domains of *epha4a* (Fig. 8N). An anterior shift of hindbrain toward the forebrain domain

is also supported by the close proximity of the hindbrain neuron and optic stalk domains of *pax2a* (Fig. 8J). Hindbrain tissue itself appears generally normal in these embryos, as indicated by r1-5 expression of *epha4a*. These results suggest that Wnt signaling is required between 7-12 hours for midbrain and MHB development. However, because heat shocks at 4.6 hpf did not produce this phenotype, and because we can infer that Wnt signaling is inhibited by that heat shock regimen until approximately 10 hpf, the 10-12 hour interval represents a critical period for Wnt-mediated midbrain/MHB development.

After a heat shock at 14 hpf, midbrain tissue appears largely normal, as judged by morphology and the distance between diencephalic and r1 expression domains of *epha4a* (19/19 transgenic embryos with phenotype) (Fig. 8C,O). While the anterior-posterior domains appear normal, expression of both *fgf8a* (4/4 transgenic embryos with phenotype) and *pax2a* (7/11 transgenic with phenotype) is absent in MHB (Fig. 8G,K), and this is accompanied by the absence of the MHB constriction (Fig. 8C). Because both morphology and marker gene expression is normal after heat shock treatments at 16 hpf (*fgf8a*: 18/18 transgenic embryos with phenotype; *pax2a*: 22/22 transgenic embryos with phenotype; *epha4a*: 17/17 transgenic embryos with phenotype) (Fig. 8D,H,L,P), these data indicate that Wnt signaling is required between 14 and 16-17 hpf for the maintenance of the MHB gene regulatory network and the MHB constriction.

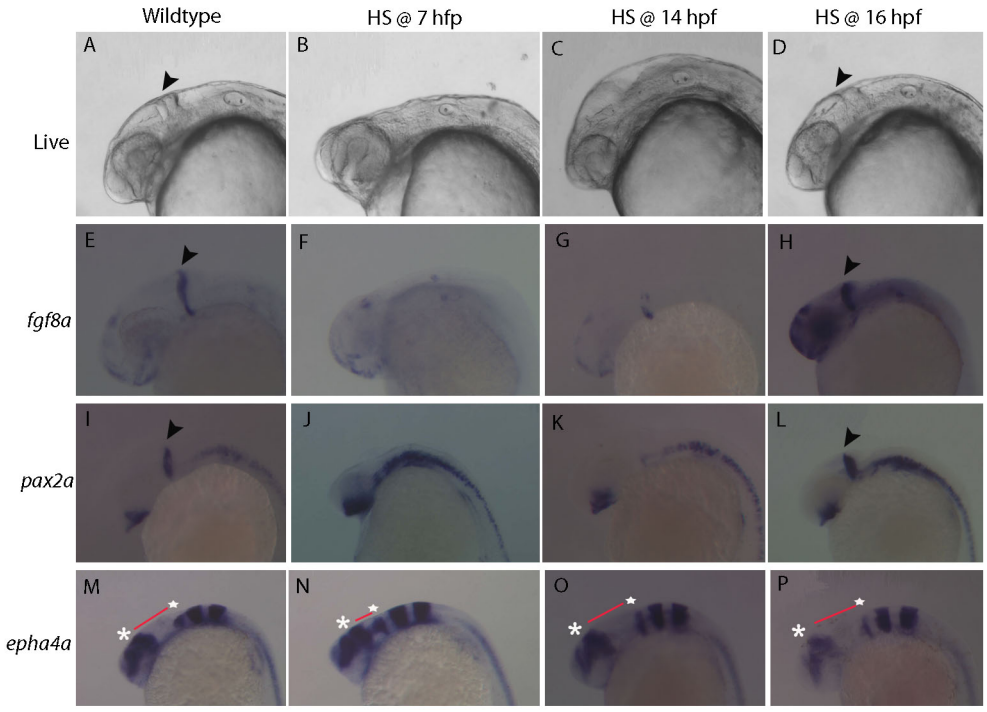

Figure 8. Inhibition of Wnt signaling at late developmental time points disrupts MHD formation. Lateral views of zebrafish heads at 24 hpf. Heat shocks at 7 hpf show MHD is interrupted (A-B). *pax2a* and *fgf8a* in the MHB are absent (MHB marked by black arrows) (E-F; I-J). The gap in *epha4a* (marked by the red line) representing MHD is reduced. (white asterick marks forebrain and white arrow marks r1) (M-N). Heat shocks at 14 hpf show midbrain and anterior hindbrain present, but MHB absent (K). *fgf8a* and *pax2a* are absent in the MHB (G,K). *epha4a* appears similar to wild type (O). Heat shocks at 16 hpf MHD appear similar to wild type (D). *fgf8a* and *pax2a* in the MHB appear similar to wild type (H,L). *epha4a* appears similar to wild type (P).

7 hpf Wnt Signaling maintains the MHB GRN during the maintenance phase

Studies in mice show deletion of *wnt1* does not affect the initial expression of *eng2a* but does affect maintenance of its expression (Danielian and McMahon, 1996). In zebrafish, *eng2a*, *wnt1*, and *pax2a* are initiated in *fgf8a* mutants, but their expression becomes reduced during somitogenesis stages (Reifers et al., 1998). Similarly, deletion of zebrafish *pax2a* does not affect activation of *wnt1* or *eng2a*, but prevent subsequent

maintenance (Lun and Brand, 1998). To examine if the inhibition of Wnt signaling affects the activation of the MHB GRN, we heat shocked embryos at this time and fixed them when these genes are first expressed and performed in situ hybridizations using *eng2a*, *pax2a*, *fgf8a*, and *wnt1*. We saw *pax2a* (6/6 transgenic with phenotype) and *fgf8a* (36/36 transgenic embryos with phenotype) are still expressed (Fig. 9C-F). *wnt1* is greatly reduced (12/12 transgenic embryos with phenotype) and *eng2a* is absent (15/16 transgenic embryos with phenotype) (Fig. 9A, B, G&H). These data suggest Wnt signaling activates *wnt1* and *eng2a*, but not *pax2a* and *fgf8a*. These data also suggest Wnt signaling activates only part of the MHB GRN.

We examined whether inhibition of Wnt signaling at 7 hpf affects the maintenance of the MHB GRN by performing heat shocks at this time and fixing them at 11.5, 14, and 16 hpf. We performed in situ hybridizations for *eng2a, fgf8a, pax2a*, and *wnt1*. *eng2a* is expressed in the MHD and is absent in heat shocked embryos when compared to wild type at these time points (11.5 hpf heat shock: 12/12 transgenic embryos with phenotype;14 hpf heat shock: 21/21 transgenic embryos with phenotype; 16 hpf: 34/34 transgenic embryos with phenotype) (Fig. 10A-D). *fgf8a* is reduced in the ventral portion of hindbrain rhombomeres r2 and r4 at 11.5 hpf in heat shocked embryos when compared to wild-type siblings (11/11 transgenic embryos with phenotype). At the 14 and 16 hpf, *fgf8a* is expanded posteriorly in the telencephalon, absent in the MHB, and reduced in the posterior neural plate in heat shocked embryos (14hpf heat shock: 15/15 transgenic embryos with phenotype; 16 hpf heat shock: 11/11 transgenic embryos with phenotype) (Fig. 10E-H). *pax2a* is reduced in the MHB and pronephric mesoderm at

11.5 hpf, and expands posteriorly in the eye field (24/24 transgenic embryos with phenotype). At 14 and 16 hpf *pax2a* is absent in the MHB and reduced in the otic placode (14 hpf heat shock: 20/20 transgenic embryos with phenotype; 16 hpf heat shock: 24/24 transgenic embryos with phenotype) (Fig. 10I-L). *wnt1* is greatly reduced in the MHB at 11.5 hpf (16/16 transgenic with phenotype), while at 14 and 16 hpf *wnt1* is reduced in the dorsal midbrain and absent in the MHB (14hpf heat shock:30/30 transgenic embryos with phenotype; 16 hpf heat shock: 16/17 transgenic) (Fig. 10M-P). These data suggest Wnt signaling regulates MHB development by maintaining the MHB GRN, and the observation of the posterior expansion of forebrain suggests a role of Wnt signaling repressing forebrain fates during gastrulation.

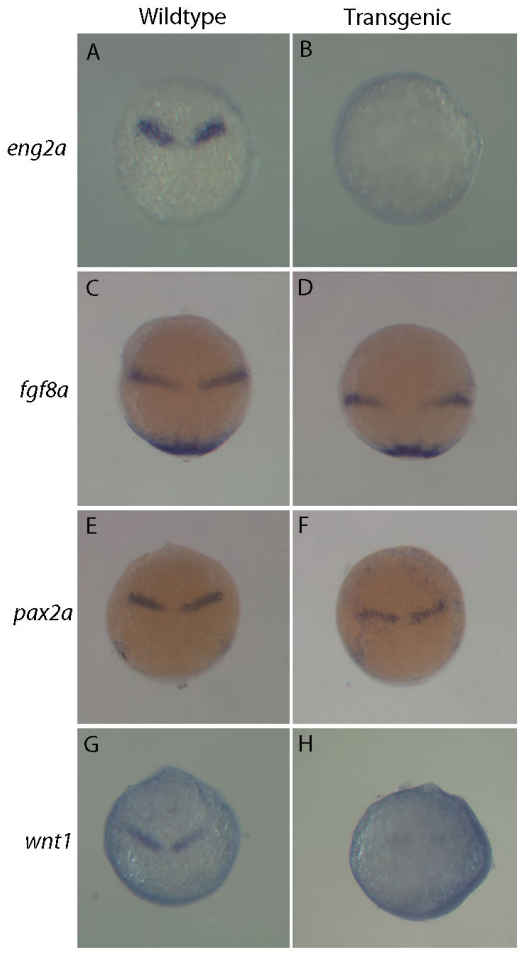

Figure 9. Wnt signaling activates *eng2a* and *wnt1*, but not *fgf8a* and *pax2a*. Dorsal views, anterior up. *eng2a* and *wnt1* in the MHD are absent or reduced respectively (A, B, G, H). *fgf8a* in the presumptive hindbrain and posterior neural plate and *pax2a* in the MHD are present though the level is lower than wild type (C-F).

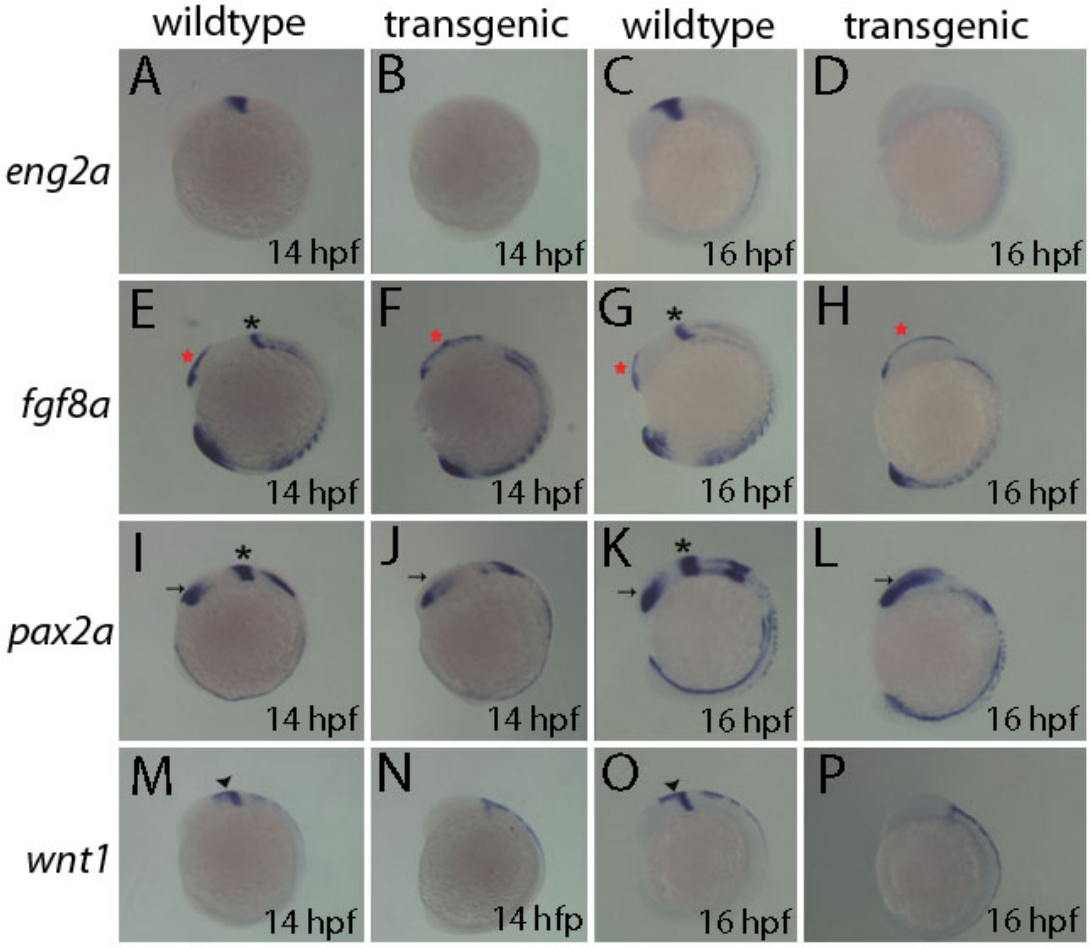

Figure 10. Wnt signaling represses forebrain, while promoting midbrain and anterior hindbrain. Lateral views of embryos with stages indicated in lower right of each panel. *eng2a* in the MHD is absent in transgenic embryos (A-D). *fgf8a* in the telencephalon (marked by the red star) extends posteriorly and is absent in the MHB (marked by the black asterisk) in transgenic embryos (E-H). *pax2a* in the optic stalk (marked by the black arrow) expands posteriorly and is absent in the MHB (marked by the black star) (I-L). *wnt1* in the MHB and dorsal midbrain are reduced (black arrowhead) (M-P).

10-12 hpf Wnt signaling represses anterior neural fate

We postulated that Wnt signaling antagonizes forebrain identity in the MHD domain during gastrulation. To test this, we performed heat shocks at 7 hpf, fixed embryos at 11.5, 14, or 16 hpf and performed in situ hybridizations with the forebrain

and midbrain markers *zic1, otx2*, and *dmbx1a*. Inhibition of Wnt signaling at 7hpf results in a posterior expansion of *zic1* (11.5 hpf heat shock: 13/13 transgenic embryos with phenotype; 14 hpf heat shock: 11 /11 transgenic embryos with phenotype; 16 hpf heat shock: 16/16 transgenic with phenotype) (Fig. 11A,B,E,F,I,J). While midbrain *otx2* in 7-hpf heat shock embryos is similar to wild type at 11.5 hpf, at 14 and 16 hpf midbrain *otx2* is reduced (11.5 hpf heat shock: 16/16 transgenic embryos with phenotype; 14 hpf heat shock: 21/21 transgenic embryos with phenotype; 16 hpf heat shock: 17/17 transgenic embryos with phenotype) (Fig 12). Consistent with the *otx2* result, midbrain expression of *dmxb1a* is reduced in heat shocked embryos at 11.5, 14 and 16 hpf (11.5 hpf heat shock: 33/36 transgenic embryos with phenotype; 14 hpf heat shock: 18/18 transgenic embryos with phenotype; 16 hpf heat shock: 13/15 transgenic embryos with phenotype) (Fig. 11C,D,G,H,K,L). These data suggest Wnt signaling may promote midbrain fate by repressing anterior neural fate.

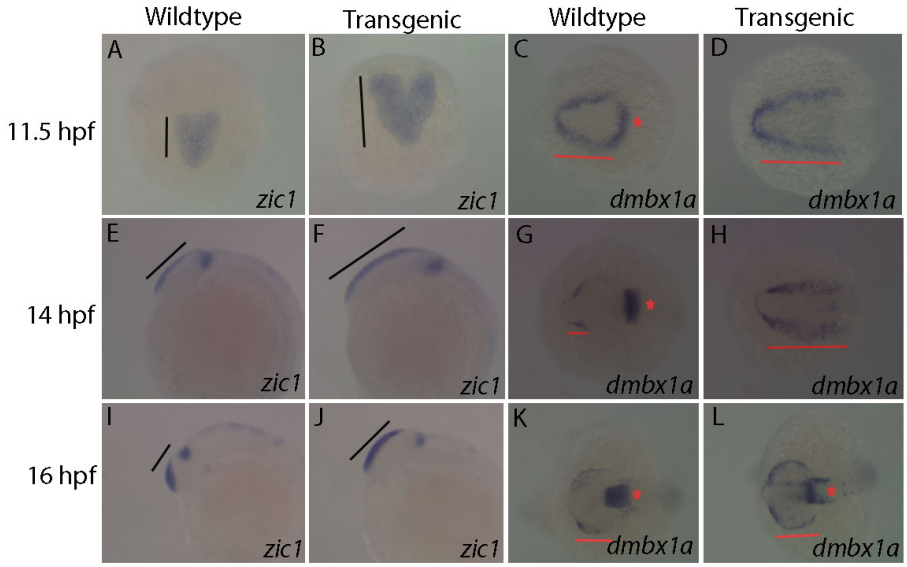

Figure 11. Wnt signaling represses forebrain while advancing midbrain development. All dorsal views except E, F, I and J which are lateral. *zic1* in the telencephalon (marked by the black line) expands posteriorly at all stages (A, B, E, F, I, J). *dmbx1a* in the forebrain expands posteriorly at all stages (marked by the red line) *dmbx1a* in the midbrain at 11.5 and 14 hpf and is absent and reduced at16 hpf (C, D, G, H, K, L).

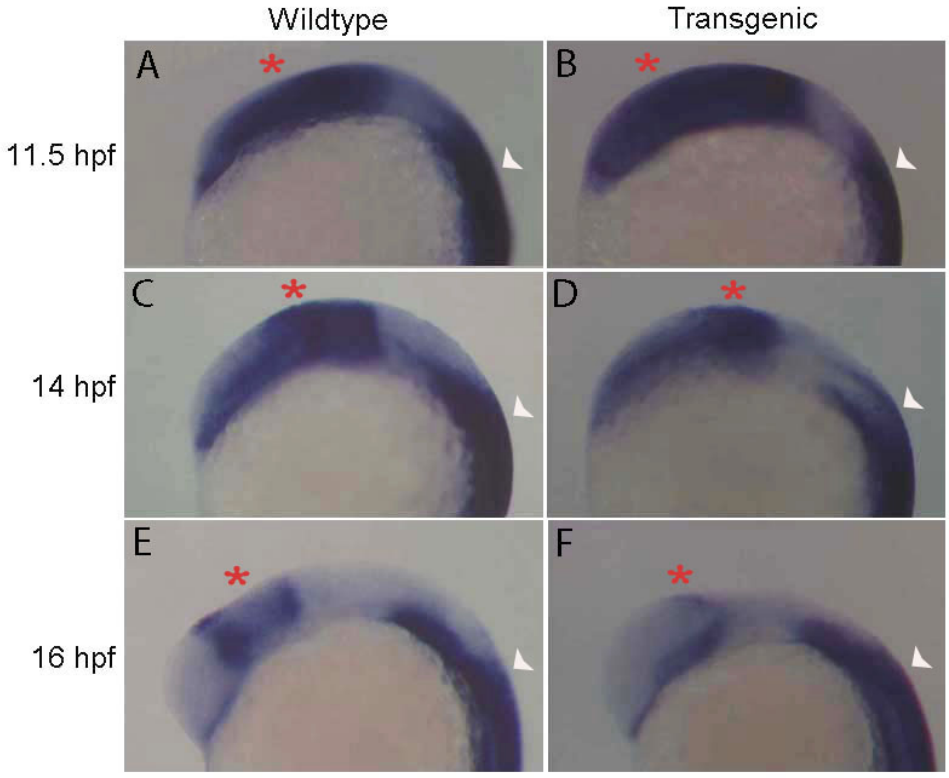

Figure 12. Wnt signaling promotes midbrain development. Lateral views of in situs for *otx2* and *hoxb1b*. *otx2* in the presumptive midbrain appears similar to wild type at 11.5 hpf (marked by red asterisk). At 14 and 16 hpf, *otx2* is reduced. *hoxb1b* in the somites and spinal cord appears similar to wild type at all stages (marked by white arrowhead).

sp5a and *sp5l* are paralogous genes previously proposed to mediate Wnt-dependent neural plate patterning (Weidinger et al., 2005). We therefore examined their expression in 7-hpf heat shocked embryos. *sp5a* and *sp5l* are expressed in the presumptive MHB and posterior neural plate at 11.5 hpf and are greatly reduced in heat shock treated embryos (*sp5a*: 11.5 hpf heat shock: 27/27 transgenic embryos with phenotype; *sp5l*: 11.5 hpf: 15/15 transgenic embryos with phenotype) (Fig. 13A,B,E,F). At 14 hpf, *sp5a* is expressed in the MHB and tailbud and is absent in heat shocked

embryos, consistent with Wnt-mediated regulation (23/23 transgenic embryos with phenotype). *sp5l* is expressed in the tailbud at this stage and is reduced (20/20 transgenic embryos with phenotype) (Fig. 13C,D,G,H) . These data are consistent with the hypothesis that Wnt signaling promotes development of midbrain, MHB and anterior hindbrain potentially through a mechanism involving *sp5a* and *sp5l*.

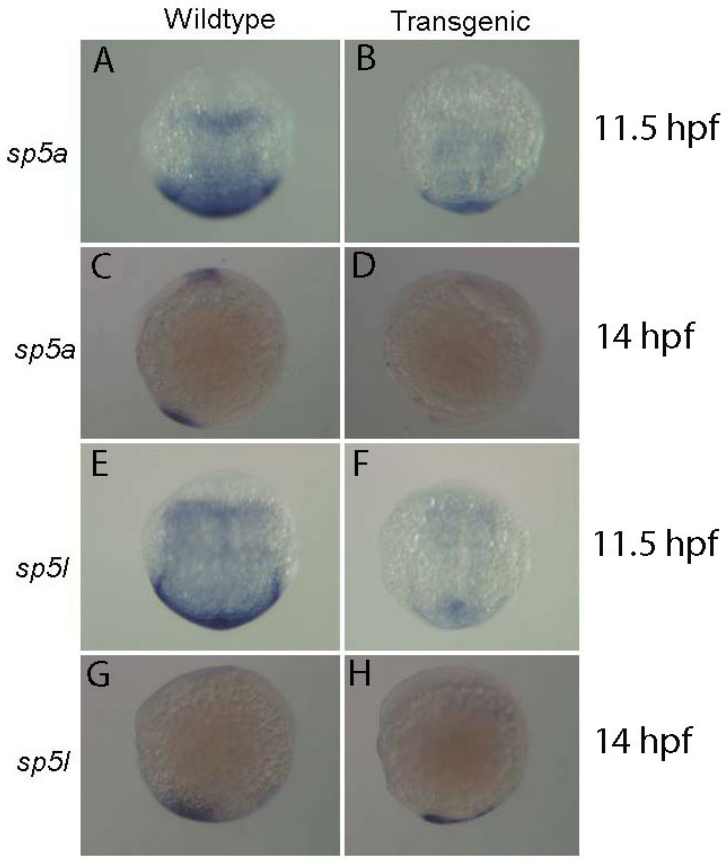

Figure 13. Wnt signaling promotes MHD formation. A, B, E, F are dorsal views at 11.5 hpf and C,D, G, H are lateral views at 14 hpf. *sp5a* and *sp5l* in the presumptive MHD and posterior neural plate are greatly reduced at 11.5 hpf. At 14 hpf is expressed in the MHD and tailbud are greatly reduced.

To directly address anterior hindbrain specification in heat shocked embryos, we assayed *gbx2*, a marker for MHB at bud stage and later (Rhinn et al., 2003). *gbx2* is slightly reduced at 11.5 hpf (12/16 transgenic embryos with phenotype) and is even more reduced at 14 hpf (19/19 transgenic embryos with phenotype). At 16 hpf, *gbx2* is expressed in the diencephalon, the expression of which shifts considerably posterior in heat shocked embryos (21/21 transgenic embryos with phenotype). Interestingly, the MHB gbx2 expression is reduced at 14 hpf, and absent at 16 hpf in heat shocked embryos, perhaps reflecting its expression in the anterior portion of the MHB and consistent with the absence of other MHB markers (Fig. 14).

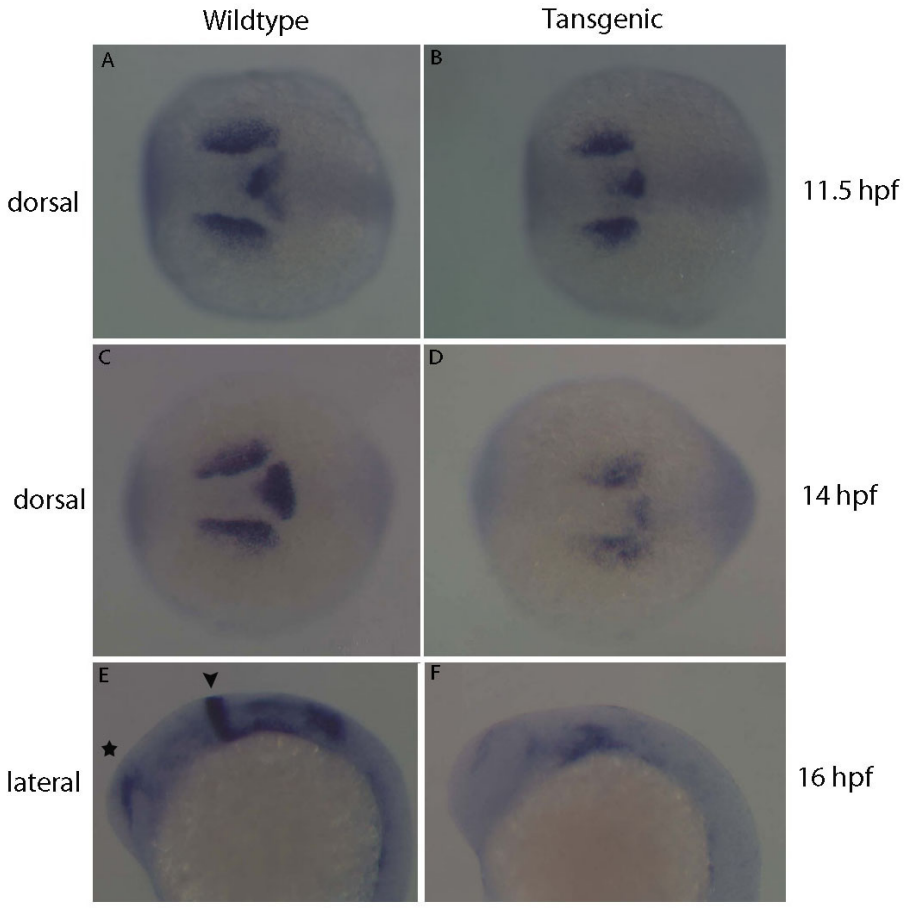

Figure 14. Wnt signaling regulates MHB and anterior hindbrain development. *gbx2* in the anterior hindbrain is slightly reduced at 11.5 hpf (A-B). At 14 hpf anterior hindbrain is more reduced (C-D). At 16 hpf *gbx2* is expressed normally in the presumptive eye (marked by black star) and MHB and are absent after heat shock (marked by black arrowhead) (E-F).

Discussion

Wnt signaling is required during later developmental stages for MHD patterning and development

In this study, we used a heat shock inducible *dkk1b* transgenic line to interfere with

Wnt signaling in defined temporal windows to probe the requirements for Wnt signaling during neural anteroposterior patterning, with a particular focus on the midbrain-hindbrain domain. We determined that a one-hour heat shock results in Wnt signaling inhibition for 5 hours. This allowed us to determine the relationship between Wnt signaling and previously proposed steps in the specification and further refinement of neural anteroposterior cell fates.

Our results show that Wnt signaling during early gastrula stages, prior to 7 hpf, establishes anteroposterior polarity that is translated into differential fate specification. Signaling at this time point is required to suppress eye field specification and establish a normal balance between forebrain, midbrain, and hindbrain territories. The next critical Wnt time window occurs between 10-12 hpf and promotes midbrain/MHB fate through the suppression of forebrain specification. The third critical Wnt time window occurs between 14-16 hpf, when Wnt signaling continues to be required for MHB GRN maintenance and MHB constriction morphogenesis.

The disruption in forebrain, midbrain and hindbrain development observed in the early gastrula heat shocks could be due to loss of *wnt8a*, the central canonical Wnt gene at these early time intervals responsible for neural posteriorization (Kishimoto et al., 1997). Studies show that *wnt8a* mutant embryos are dorsalized (Lekven et al., 2002), which we see with inhibition of Wnt signaling at 3 hpf. When Wnt signaling is blocked at 4.3 and 4.7 hpf the effects of dorsalization are less severe, but the patterning deficiencies are similar to 3 hpf heat shocks. These results are consistent with chick studies, in which prospective forebrain explants exposed to progressively greater

concentrations of Wnt signaling produced more caudal neural fates like midbrain and anterior hindbrain in a graded fashion (Nordstrom et al., 2002). Although there is mispatterning of the brain in our early heat shock embryos, the fact that these structures form at 24 hpf indicates that Wnt signaling is not required during gastrulation for their specification, raising the question of what signals or factors are responsible for imparting anteroposterior identity. One possibility is Fgf signaling, as multiple Fgf genes are expressed in the embryonic margin during this time window and are known to regulate anteroposterior patterning (Sato et al., 2004).

Inhibition of Wnt signaling at 7 hpf results in a partial activation of the MHB GRN which is not maintained

The most severe phenotype was observed upon 7 hpf heat shocks, likely due to a requirement for Wnt signaling in the midbrain-hindbrain domain during the 10-12 hpf time window. Prior studies established that the MHB GRN is activated but not maintained in *wnt1;wnt10b*, *pax2a*, and *fgf8a* mutants (Lekven et al., 2002), leading to the hypothesis that multiple independent mechanisms establish *wnt1*, *fgf8a* and *pax2a* expression in the MHD. In agreement with these studies *pax2a* and *fgf8a* are activated but not maintained in 7 hpf heat shock embryos. Interestingly *eng2a* is never activated in these embryos, consistent with being a direct Wnt target gene (McGrew et al., 1995). Although studies in Xenopus proposed that *eng2a* can be activated by *fgf*, *wnt1,* and *wnt3a* either directly or indirectly (Merzdorf and Sive, 2006), our results suggest that Fgf, if it has a role in *eng2a* regulation, may not be able to compensate for global Wnt inhibition. Our observation that *wnt1* is greatly reduced in 7 hpf heat shock embryos

suggests an autoregulatory mechanism. *wnt1* activation is not well understood, though it is suggested *pax2a* may play a role (Favor et al., 1996).

The severe phenotype of 7 hpf heat shock embryos can be explained by the rapid and progressive expansion of forebrain identity into the midbrain primordium. *pax2a* and *fgf8a* expression is initially normal in 7 hpf heat shock embryos, but forebrain expression domains expand posteriorly, consistent with a requirement for Wnt signaling in their repression. Previous studies of mouse *wnt1* knockouts demonstrated that the loss of midbrain and anterior hindbrain tissue could be attributed to the progressive loss of *eng* gene expression, since driving *eng* with *wnt1* regulatory elements could largely rescue *wnt1* loss of function (Danielian and McMahon, 1996). Our data are consistent with this being a conserved mechanism in vertebrates.

Our experiments further clarified a critical time window during which Wnt signaling is essential for maintenance of the Wnt-*pax2a-fgf8* cross regulatory network. We previously showed that zebrafish *wnt3a*;*wnt1*;*wnt10b* mutants lose *pax2a* and *fgf8a* expression from the ventral MHB by approximately 15 hpf, but those experiments could not define the time period that Wnt signaling was required for this function (Buckles et al., 2004). It is still unclear how Wnt signaling maintains *pax2a* and *fgf8* expression, but identifying this developmental window will require further experiments to define this interaction.

CHAPTER IV

IS *WNT3* FUNCTIONAL ROLE IN MHB FORMATION SEPARATE FROM *WNT3A*, *WNT1*, AND *WNT10B*

Introduction

Wnt signaling is involved in many developmental and cellular processes including cell proliferation, apoptosis, and anterior posterior patterning of vertebrate embryos. In the developing vertebrate brain, Wnt signaling is required for the formation of the midbrain-hindbrain boundary (MHB), an evolutionarily conserved signaling center that patterns the adjacent midbrain and hindbrain (Raible and Brand, 2004). Despite a common requirement in vertebrates for Wnt signaling in MHB formation, different ligands are required in the taxa in which this has been investigated. *wnt1* is required in mouse, but *wnt1*, *wnt10b* and *wnt3a* are required in zebrafish (Danielian and McMahon, 1996; Buckles et al., 2004). One additional ligand, *wnt3*, is known to be expressed in the MHB, and *wnt3* functions redundantly with its paralog, *wnt3a*, in several developmental contexts (Mattes et al., 2012; Clements et al., 2009). This study addresses the question of whether zebrafish *wnt3* functions redundantly to *wnt3a* in MHB development, and whether *wnt3* relationship with *wnt1* and *wnt10b* reflects a similar relationship of *wnt3a*, *wnt1* and *wnt10b*.

In the mouse, *wnt3* and *wnt3a* are expressed in the dorsal neural tube, forebrain, and midbrain (Roelink and Nusse 1991). *wnt3* functions in mice to maintain the epiblast during gastrulation but is not involved in the induction of gastrulation (Tortelote et al.

2013; Liu et al., 1999), which has made studying *wnt3* functions in the neural tube difficult to carry out. In contrast, *wnt3a* stimulates cell proliferation in the developing hippocampus (Lee et al., 2000) and functions redundantly with *wnt1* in the neural tube, as demonstrated by the fact that double mutants have smaller hindbrains and severely affected dorsal spinal cords (Ikeya et al., 1997).

In the chick embryo, *wnt3a* is expressed in the presumptive rhombencephalon and later in the dorsal neural tube and spinal cord and midbrain (Hollyday et al., 1995). *wnt3* expression is observed in the forebrain, midbrain, and hindbrain. It is expressed in the spinal cord and this expression increases as the embryo develops (Robertson et al., 2004). Studies show that *wnt1* and *wnt3a* produce a mitogenic gradient during spinal cord development (Megason and McMahon, 2002). *wnt1* and *wnt3a* are also needed for the development of dorsal interneurons, specifically D1 and D2 (Muroyama et al., 2002). Explant culture assays performed in chick suggest *wnt3* plays a role in neural crest proliferation and *wnt3a* in melanocyte development (Dongkyun et al., 2010).

In Xenopus, *wnt3a* is first observed in portions of the neural folds, which will become the head. It is observed along the anterior posterior axis especially in the brain at further stages in the developing embryo. At later stages in development, expression is observed in the forebrain and midbrain (Wolda et al., 1992). In animal caps expressing Wnt3a and the BMP antagonist Noggin, a decrease in expression levels of posterior neural markers and an increased anterior neural marker expression is observed (Dibner et al., 2001).

In zebrafish, *wnt3a* and *wnt3* are paralogs that are both expressed in the MHB and anterior hindbrain, suggesting they may have redundant functions in brain development. *wnt3* and *wnt3a* are both needed for patterning the mid-diencephalic organizer (MDO) (formerly the zona limitans intrathalamica (ZLI)) through inhibition of cell death (Mattes et al., 2012). There are differences in the expression patterns of *wnt3* and *wnt3a*, which raises the possibility these genes may not have redundant functions in MHD formation. *wnt3a* is expressed in the midbrain and spinal cord but is only expressed in the dorsal portion of the MHB (Buckles et al., 2004). *wnt3* is expressed in the ZLI, basal plate, and MHB (Clements et al., 2009). While the role of *wnt3a* in MHB development has been examined, it is unknown whether *wnt3* is required in this process, and whether it shows an interaction with *wnt1* and *wnt10b*.

Loss of *wnt1*, *wnt10b,* and *wnt3a* produces a complete absence of the MHB, suggesting these genes act cooperatively in MHB development (Buckles et al., 2004). Buckles et al. (2004) also showed that genes involved in regulating MHB development, *eng2a, pax2a, and fgf8a* are absent when *wnt3a* is knocked down along with *wnt1* and *wnt10b* (Buckles et al., 2004). *wnt3a*, *wnt1*, and *wnt10b* are activated at 8-9 hpf, while *wnt3* is activated at 11.5 hpf (Buckles et al., 2004, Lekven et al. 2003, Clements et al., 2009). If *wnt3a*, *wnt1*, and *wnt10b* are activated at early stages and regulate MHB development cooperatively, then what is the role of *wnt3* in the process since it comes on later but has overlapping expression with the other Wnt genes?

In this chapter, we dissect the role of *wnt3* in MHB development in cooperation with *wnt1*, *wnt3a*, and *wnt10b*. We find that *wnt3a* appears to have a greater effect on

MHB formation, likely reflecting a requirement for neural plate expression at 10 hpf to specify the midbrain-hindbrain domain. In contrast, *wnt3* appears to function during segmentation stages in tandem with *wnt1* and *wnt10b* to promote formation or maintenance of the MHB morphological constriction.

Materials and Methods

Zebrafish care

Zebrafish were maintained as described (Westerfield, 2000). An AB-TL hybrid line serves as our wild-type stock. Vertebrate animal procedures were approved by the Texas A&M Institutional Animal Care and Use Committee. The *wnt1;wnt10b* deficiency allele is $Df(LG23)wnt1^{w5}$. $pax2a^{tu29a}$ and $fgf8a^{X15}$ mutant lines were also used.

In situ hybridizations

The protocol we use for single in situ hybridizations is adapted from Oxtoby and Jowett (1993). Fixed embryos are dechorionated in PTW (1X PBS, 0.1% Tween-20) and stored in methanol at -20°. Embryos are rehydrated by a series of washes including a 1X wash of 50% methanol/50% PTW for 5 minutes and next 2-3 washes of PTW for 5 minutes. Embryos were proteinase K treated with 1 ul 10mg/ml proteinase K/1 ml PTW. Embryos at somite stages are treated for 5-10 minutes and 24 hpf embryos are treated for 20-25 minutes and then washed 2X with 1 mg/ml glycine/ 50 ml PTW. Embryos are fixed again in 4% paraformaldhyde for 20 minutes and washed 2X with PTW for 5 minutes. Embryos are placed in a hybridization buffer (50% formamide, 5X SSC, 0.1% Tween-20; 50ug/ml heparin, 500 ug/ml tRNA and 5% dexatran sulfate) at 67° for an hour to prehybridize the embryos. After this a digoxigenin labeled RNA probe (0.5

ng/ul-1ng/ul) is placed on the embryos overnight at 67°.

The next day embryos are washed in a series of graded washes to remove excess probe. Beginning with 2X at 67° for 30 minutes with 2X SSC, 50% formamide, and 0.1% Tween-20, then once for 15 minutes at 67° with 2X SSC, and 0.1% Tween-20 and last 2X at 67° for 30 minutes with 0.2X SSC, and 0.1% Tween-20. Embryos are washed with PTW for 5 minutes at room temperature 2X and placed in a blocking solution (1.5 ml FBS in PTW for 15 ml total) for an hour. After this initial blocking step embryo are incubated for either 2 hours at room temperature or overnight at 4° in antibody (1:2000 for 2 hours, 1:5000 overnight).

After the antibody treatment, embryos are washed for 15 minutes 4X in PTW. Embryos are placed in a staining buffer (100mM Tris pH 9.5; 50nM MgCl2, 100nM NaCl; 0.1% Tween-20) for 5 minutes and then in a color reaction solution of 4.5 ul/ml NBT and 3.5 ul/ml BCIP in staining buffer and stopped after reaction using methanol. Embryos then are placed in a graded glycerol series and kept in 70% glycerol.

The probes *pax2a, eng2a, fgf8a* that are expressed in the MHD were used (Krauss et al., 1991; Fjose et al., Haffter et al., 1996). *otx2* expressed in the presumptive midbrain was used (Li et al., 1994). Also, *atoh1a* and *epha4a*, which are expressed in the hindbrain were used (Kim et al., 1997; Xu et al., 1994). *wnt1, wnt10b, wnt3*, and *wnt3a* which are expressed in the MHD were used (Molven et al., 1991; Krauss et al., 1992; Clements et al., 2009).

Morpholino design and injection

The *wnt3a* morpholino was previously described (Buckles et al., 2004). The *wnt3*

morpholino was previously described (Mattes et al., 2012). The *wnt3* MO (5 mg/ml) and *wnt3a* MO (2mg/ml) were injected either together or separately in 1-2 cell embryos from intercrosses of either Df^{w5}/+ adult fish or wild-type adult fish. Embryos were raised at 29° and assayed at 24 hpf for MHB morphology. Embryos were also fixed in 4% paraformaldhyde overnight at 4° at somite stages or 24 hpf.

pax2a and fgf8a crosses

Adult zebrafish containing the *fgf8a* or *pax2a* mutation were intercrossed. Embryos were collected and fixed at 10 somite stage in 4% paraformaldhyde overnight at 4°C.

Results

wnt3 and wnt3a functionally interact differently when combined with wnt1;wnt10b

The overlapping but distinct expression patterns of *wnt3* and *wnt3a* raises the question of whether they are functionally redundant in promoting MHB development. To evaluate this possibility, we used a morpholino antisense oligonucleotide-based approach to reduce *wnt3* and *wnt3a* expression in wild-type, Df^{w5}/+, or Df^{w5}/Df^{w5}embryos (hereafter called Df^{w5} embryos), which carry a deficiency allele of the linked *wnt1* and *wnt10b* loci (Lekven et al., 2003). In previous studies, we observed that morpholino (MO) knockdown of *wnt3a* in embryos derived from Df^{w5}/+ intercrosses altered the phenotype only of Df^{w5} homozygotes, that is, ~25% of embryos displayed a MHB phenotype, with ~75% of injected embryos expressing the wild-type phenotype, which we deduce to comprise both +/+ and Df^{w5}/+ embryos (Buckles et al., 2004). Thus, Df^{w5}/Df^{w5}embryos are sensitized to changes in expression of additional Wnt genes in the MHB. Because Df^{w5}/Df^{w5} embryos represent a sensitized system, we infer that similar

experiments that produce phenotypes in ~25% of injected embryos derived from intercrosses of *Df*^{w5}/+ fish reveal interactions in MHB development and that the ~25% class represents *Df*^{w5} homozygotes, while the ~75% fraction comprises +/+ and *Df*^{w5}/+ embryos that received the injection. In the following experiments, reported phenotypes are observed in ~25% of injected embryos.

We injected *wnt3* or *wnt3a* morpholino (MO) either alone or together into embryos collected from an intercross of *Df*^{w5}/+ carrier fish, and then examined embryos at 24 hpf. Inhibition of either *wnt3* or *wnt3a* in wild-type or *Df*^{w5}/+ heterozygotes resulted in normal appearing MHBs (Fig. 15A-C; *wnt3*MO: 35/47 injected embryos from cross; *wnt3a*MO:23/31 injected embryos from cross). Knockdown of both *wnt3* and *wnt3a* in wild-type embryos resulted in absence of MHB in a small percentage of embryos (Fig. 15D). In *wnt3* MO-injected *Df*^{w5} embryos, the MHB is significantly shallower when viewed dorsally compared to *Df*^{w5} embryos (Fig. 15E,F; 12/47 injected embryos from cross). When both *wnt3* and *wnt3a* are injected into *Df*^{w5} embryos, the MHB is absent (Fig. 15G; 14/45 injected embryos from cross). These data suggest that *wnt3* and *wnt3a* appear to be redundant for MHB formation, though they have different functional relationships to *Df*^{w5} mutants.

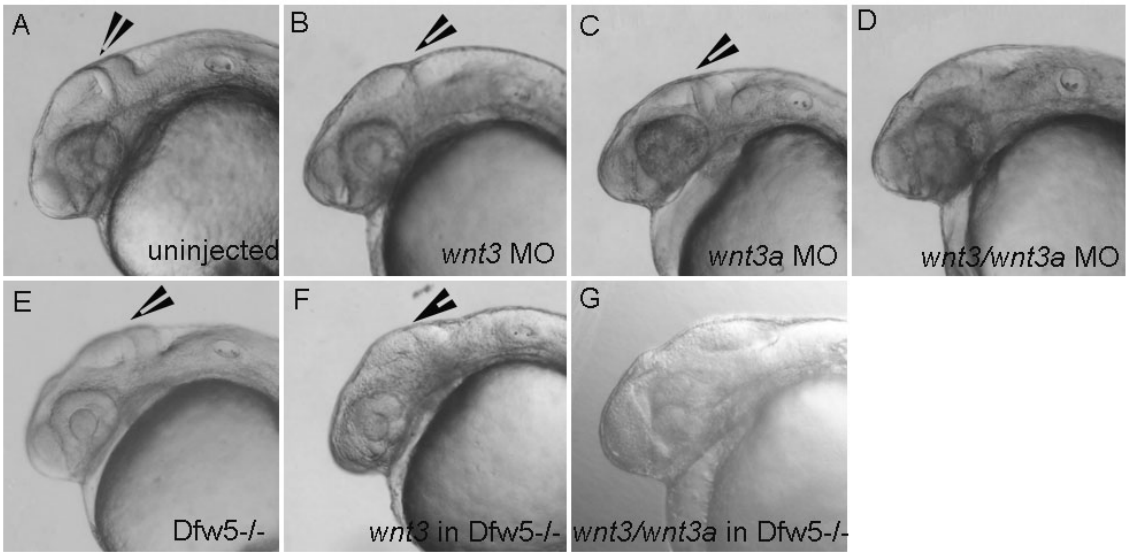

Figure 15. Cooperatively of Wnt function in MHD development. Lateral views of heads of 24 hpf live embryos. Morpholinos used and recipient genotypes are indicated for each panel. The MHB appears morphologically normal in *wnt3a* morphants, *wnt3* morphants and Df^{w5} homozygotes (A, B, C, E). *wnt3/wnt3a* knockdown results in MHB constriction absence (D). *wnt3* knockdown in Df^{w5} embryos results in a shallow constriction (F). *wnt3/wnt3a* knockdown in Df^{w5} embryos results in MHB absence.

wnt3 and wnt3a regulate fgf8a at somitogenesis stages.

We examined whether *wnt3* and *wnt3a* have different functions in MHB GRN regulation. We injected *wnt3* or *wnt3a* MOs either alone or together into embryos collected from an intercross of Df^{w5} carrier fish, and then performed in situ hybridizations on somite staged embryos using *pax2a, eng2a, fgf8a*, and *otx2*. Studies show *pax2a*, *eng2a*, *fgf8a* are required for MHB development and are expressed in the MHD (Lun and Brand, 1998; Reifers et al., 1998; Zervas et al., 2005). *otx2* is expressed in the presumptive midbrain and required in midbrain development and positioning the MHB (Rhinn et al., 2005). MHB *pax2a* and *fgf8a* are similar to uninjected, except for *wnt3* and *wnt3a* MO injected Df^{w5} embryos, in which *pax2a* is reduced and *eng2a* is

restricted to the dorsal MHB (Fig. 16A-H). *fgf8a* in the MHB is reduced or absent when *wnt3* and *wnt3a* are blocked in wild-type (Fig. 16K) or Df^{w5} embryos (Fig. 16L). *otx2* in the midbrain is similar to uninjected embryos (Fig. 16M-O; no differences observed with *wnt3*MO injection, n=20, *wnt3a*MO injection, n=24, or co-injection, n=22). These data suggest both *wnt3* and *wnt3a* regulate *fgf8a* at somite stages but not *pax2a* and *eng2a*. These data suggest that the Wnt genes under study have differential requirements in regulating MHB formation.

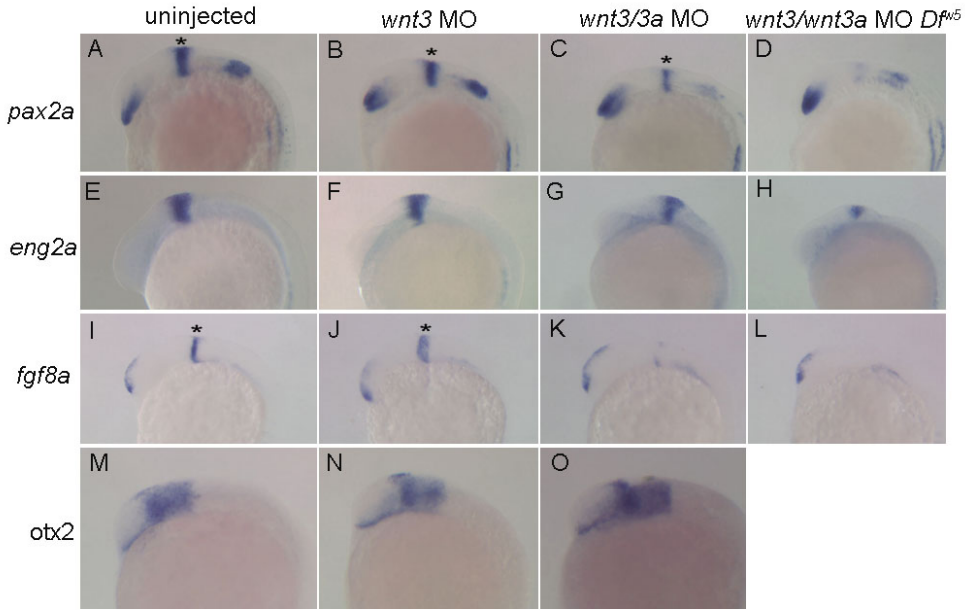

Figure 16. Cooperation of Wnt genes regulates MHB regulatory genes during somite stages. Lateral views of heads of 14-16 somite stage (A-L) or 10 somite stage (M-O) embryos. *wnt3* and *wnt3a* have a weak effect on *pax2a* and *eng2a* expression (A-H). *wnt3* and *wnt3a* appear to have a stronger effect on *fgf8a* expression (I-L). Midbrain size appears normal at somite stages (M-O).

Wnt genes regulate MHD development at later stages

We examined whether *wnt3* and *wnt3a* have different functions in MHD regulation at 24 hpf. We injected *wnt3* or *wnt3a* MOs either alone or together into embryos

collected from an intercross of *Df*^{w5} carrier fish, and then performed in situ hybridizations on somite staged embryos using *pax2a, eng2a, fgf8a*, *atoh1a* and *epha4a.* *atoh1a* and *epha4a* play roles in cerebellar development. *atoh1a* and *epha4a* are expressed in the hindbrain (Jászai et al., 2003). *pax2a, eng2a,* and *fgf8a* are restricted to the dorsal MHB in *wnt3MO*-injected *Df*^{w5} embryos (Fig. 17D,I,N), while *eng2a* and *pax2a* are absent in *wnt3;wnt3aMO*- injected wild-type embryos (Fig. 17C,H). Interestingly, *fgf8a* expression in *wnt3;wnt3a* MO injected embryos is similar to uninjected (Fig. 17M). *eng2a*, *fgf8a*, and *pax2a* in the MHB are absent in *wnt3*;*wnt3aMO*-injected *Df*^{w5} embryos (Fig. 17E,J,O). *atoh1a* in the rhombic lip of r1 gradually decreases as additional Wnt genes are inhibited (Fig. 17P-T). The gap in *epha4a* representing the MHD decreases as more Wnt genes are blocked (Fig. 17U-Y). These data suggest *wnt3a* is needed to regulate *pax2a*, *eng2a*, and *fgf8a* in the dorsal MHB, while *wnt3* in combination with *wnt1* and *wnt10b* regulate ventral MHB. These data therefore suggest *wnt3, wnt3a, wnt1*, and *wnt10b* regulate MHD formation cooperatively in the dorsal and ventral domains.

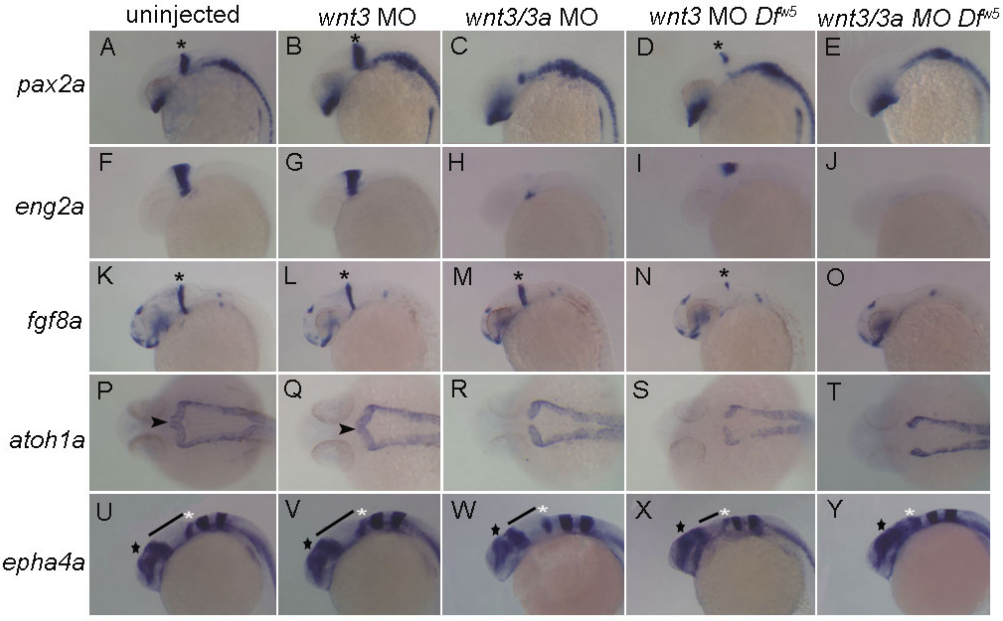

Figure 17. Cooperation of Wnt genes regulates MHD patterning at 24 hpf. Lateral views of heads of 24 hpf embryos, except P-T, which are dorsal. *wnt3* alone does not affect *pax2a, eng2a* or *fgf8a* (A, B, F, G,K,L). *wnt3* in combination with *wnt1* and *wnt10b* affect ventral MHB formation (D,I,N). *wnt3* and *wnt3a* regulate *pax2a* and *eng2a* expression but not *fgf8a* expression (C,H,M). Together all four genes regulate *pax2a, eng2a*, and *fgf8a* cooperatively in MHB development (E, J,O). *wnt3* alone does not affect *atoh1a* expression, but reduces *atoh1a* expression when *wnt3* is injected in Df^{w5} embryos (P, Q,S). *wnt3* and *wnt3a* cause a slight reduction in *atoh1a* expression, while *atoh1a* expression is greatly reduced in combination with *wnt1* and *wnt10b* (R,T). *wnt3* alone does not affect *epha4a* expression but along with *wnt1* and *wnt10b* reduces the gap between the forebrain and cerebellum. *wnt3* and *wnt3a* also reduce the gap between the forebrain and hindbrain and this gap is absent when all four Wnt genes are knocked down (U-Y).

Wnt genes regulates MHB development differently

Our data show that *wnt3* and *wnt3a* regulate MHB formation differently in combination with *wnt1* and *wnt10b*. When we inhibit *wnt3*, *wnt1*, and *wnt10b* we observe a partial loss of the MHB constriction, and previous studies showed that inhibiting *wnt3a*, *wnt1*, and *wnt10b* causes a complete loss of the MHB (Buckles et al.,

2004). Our findings also show that inhibition of *wnt3a* and *wnt3* leads to loss of the MHB, though with a less penetrance than *wnt3a-wnt1-wnt10b* knockdown. Therefore, we postulated that Wnt genes might function in a hierarchy to regulate MHB development with *wnt3a* occupying a more superior position in the hierarchy, and *wnt1*, *wnt3*, and *wnt10b* occupying more inferior positions. To examine this question, we injected *wnt3* or *wnt3a* MOs either alone or together into embryos collected from an intercross of Df^{w5} carrier fish or an intercross of wild-type fish, and then performed in situ hybridizations on 24 hpf embryos using *wnt1, wnt3* and *wnt3a. wnt1* is present in *wnt3* MO-injected wild-type embryos (Fig. 18B) but is absent in the MHB of *wnt3*;*wnt3a* MO injected wild-type embryos (Fig. 18C). Dorsal midbrain *wnt1* expression persists in knockdown embryos, indicating that this expression domain is independent of *wnt3* and *wnt3a*. *wnt3a* expression in *wnt3* MO injected Df^{w5} embryos appears similar to expression in wild-type, but *wnt3a* is normally only expressed in the dorsal midbrain midline and is not expressed in the mature MHB (Fig. 18D,E). Thus, *wnt3a* expression is not normally observed in the mature MHB. *wnt3* is absent from the MHB and basal plate of *wnt3a* MO injected Df^{w5} embryos (Fig. 18F,G). These data suggest Wnt genes form a hierarchy during MHB development with *wnt3a* playing a superior role and *wnt1*, *wnt10b*, and *wnt3* playing inferior roles.

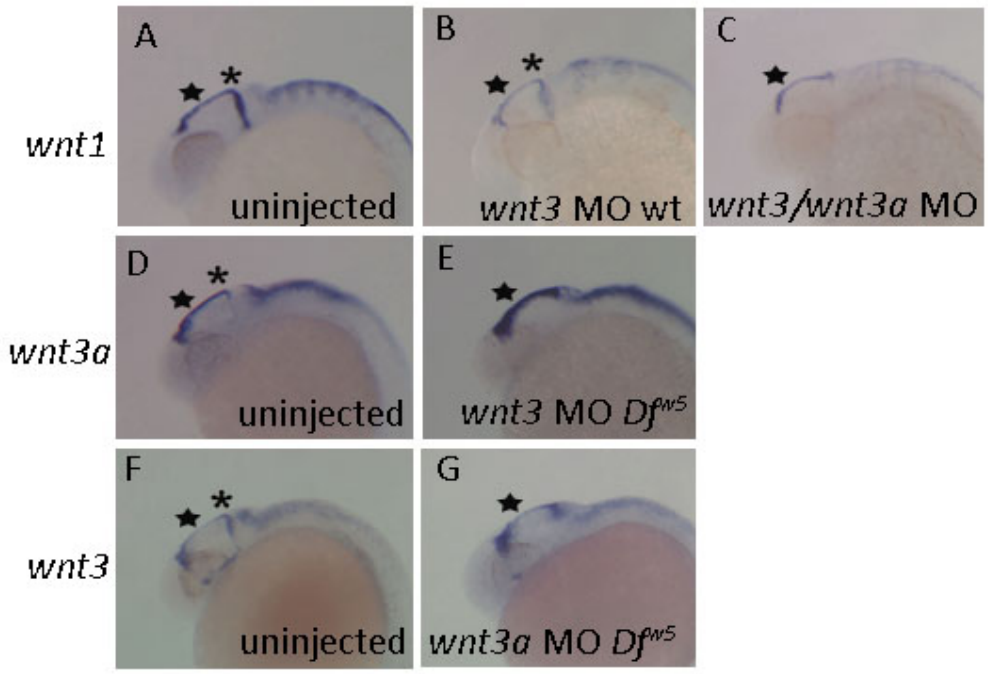

Figure 18. Wnt genes form a hierarchy to regulate MHB formation. Lateral views of heads of 24 hpf embryos. MO injected and recipient embryo genotype are indicated in each panel, probes used indicated to the left. *wnt1* expression is present in the MHB when *wnt3* is blocked, but absent in *wnt3* and *wnt3a* knock downs (A-C). *wnt3a* expression is missing in the MHB in *wnt3* MO injected Df^{w5} and *wnt3* expression is absent in the MHB in *wnt3a* MO injected Df^{w5} (D-E).

pax2a and fgf8a regulate Wnt genes at somitogenesis stages

We determined that *wnt3* and *wnt3a* regulate *fgf8a* but not *pax2a* during somitogenesis stages, which led us to ask if *pax2a* and *fgf8a* recriprocally regulate Wnt genes during this time. We collected embryos from *noi (pax2a)* or *x15 (fgf8a*) mutants and performed in situ hybridizations on 10 somite stage embryos using *wnt1*, *wnt3*, *wnt3a*, and *wnt10b*. *wnt3, wnt3a, wnt1*, and *wnt10b* in the MHB are absent in both *noi* and *x15* mutants (Fig. 19A-I). These data suggest *fgf8a* and *pax2a* paly a role in

regulating Wnt genes during somitogenesis in MHB development.

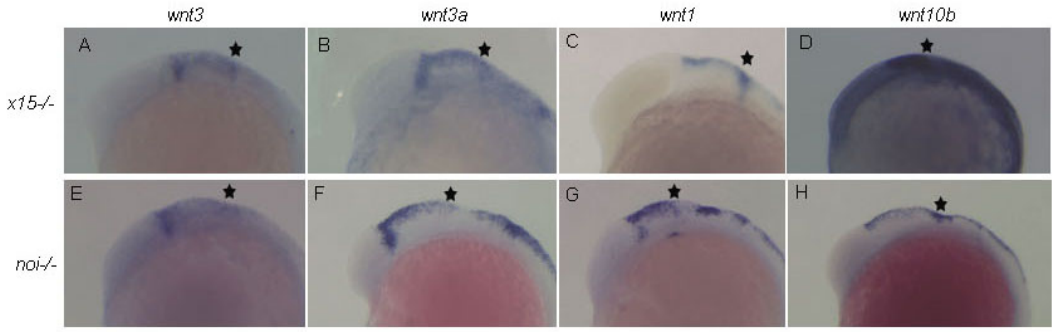

Figure 19. *fgf8a* and *pax2a* regulate Wnt genes during MHB formation. Lateral views of zebrafish heads at 10 somite stage in the *x15* and *noi* mutants. *wnt3*, *wnt3a, wnt1,* and *wnt10b* expression are absent in the MHB in *x15* and *noi* mutants.

Discussion

Wnt genes work cooperatively in MHD development

In this study we use *wnt3* and *wnt3a* morpholinos to knock down expression of these genes in Df^{w5} embryos, which lack *wnt1* and *wnt10b*, to examine the role of these genes in MHB formation.

Our results show both *wnt3* and *wnt3a* appear to act redundantly in MHB formation though their roles in combination with *wnt1* and *wnt10b* are different. *wnt3a* in combination with *wnt1* and *wnt10b* has a greater effect on MHB development than *wnt3* in combination with *wnt1* and *wnt10b*. When *wnt3, wnt1*, and *wnt10b* are blocked the MHB constriction partially forms at 24 hpf. Studies previously performed in this lab showed *wnt1*, *wnt10b*, and *wnt3a* work in combination with each other during MHB development (Buckles et al., 2004). When observe that *wnt3* and *wnt3a* inhibition leads

to MHB absence. We postulate that *wnt3a* has a greater effect on MHB development since *wnt3a* inhibition is required in any combination of knockdowns to generate the most severe patterning phenotypes. *wnt3* and *wnt3a* are paralogs and have redundant functions in MDO formation (Mattes et al., 2012). These data suggest *wnt3* and *wnt3a* function differently in combination with *wnt1* and *wnt10b*, with *wnt3a* playing a superior role in MHB development.

wnt3 and *wnt3a* are not required for *pax2a* and *eng2a* regulation, but do regulate *fgf8a* during somitogenesis. At somite stages, *pax2a* and *fgf8a* are reduced and *eng2a* is restricted to the dorsal MHB in *wnt3a* MO injected Df^{w5} embryos, suggesting these three genes maintain the MHB GRN (Buckles et al., 2004). Our results show a similar phenotype when *wnt3* and *wnt3a* are inhibited in Df^{w5} embryos, but when *wnt3* is blocked in Df^{w5} embryos *pax2a, fgf8a*, and *eng2a* expression are similar to uninjected. These suggest that *wnt3* function during somite stages is not to regulate the MHB GRN but *wnt3a*, *wnt1*, and *wnt10b* do regulate MHB development at this stage. These data also suggest a differential requirement for Wnt genes during MHB development at this stage.

Midbrain *otx2* is not affected by inhibition of the Wnt genes under study, suggesting that Wnts are not required for midbrain patterning at this stage. A possibility for this result is other pathways, such as FGF, are able to compensate for the absence of Wnt genes. Studies show FGF regulates in midbrain development (Sato et al., 2004). Another possibility is Wnt signaling is a mitogen and at this stage Wnt genes play a role in cell proliferation and not midbrain patterning.

At 24 hpf in development, *wnt3* and *wnt3a* have separate functions in MHB formation. Buckles et al. (2004) showed that inhibition of *wnt3a*, *wnt1*, and *wnt10b* resulted in an absence of *pax2a* and *fgf8a* in 24 hpf embryos (Buckles et al., 2004). We show MHB GRN is restricted to the dorsal MHB in *wnt3* MO injected Df^{w5} embryos suggesting *wnt3* regulates ventral MHB but is not needed for dorsal MHB formation. One possibility is that *wnt3a* is expressed in the dorsal MHB, while *wnt3* is expressed throughout the MHB (Clements et al., 2009). *wnt3a* could regulate dorsal MHB patterning, and since it is not inhibited it may compensate for the loss of *wnt3* in Df^{w5} embryos. When *wnt3a* is blocked along with these three other Wnt genes, there is a complete loss of MHB suggesting *wnt3, wnt3a*, *wnt1* and *wnt10b* work in combination at later stages in development to regulate MHB formation. These data also suggest there is a differential requirement for Wnt genes in MHB development.

A curious observation is that *fgf8a* expression is reduced in *wnt3* and *wnt3a* morpholino-injected embryos during somite stages, but appears similar to uninjected at 24 hpf. One explanation could be that *wnt3* and *wnt3a* are required for *fgf8a* regulation at somite stages but not at later stages in development. At 24 hpf in development *fgf8a* could be regulated by another pathway, though which pathway would be responsible for this effect is not clear. Further studies need to be performed to explain this inconsistency.

In *wnt3* MO injected Df^{w5} embryos, we observe an anterior expansion of anterior hindbrain. Studies have shown that blocking *wnt1*, *wnt10b*, and *wnt3a* expands anterior hindbrain and this tissue is respecified into midbrain (Buckles et al., 2004). It is possible

that *wnt3* in combination with *wnt1* and *wnt10b* is needed to maintain the morphological structures of the MHD. More experiment need to be performed to examine this possibility.

wnt genes form a hierarchy during MHB formation

We observe that blocking *wnt3* in combination with *wnt1* and *wnt10b* does not have as severe an effect as knockdown of *wnt3a* with *wnt1* and *wnt10b* (Buckles et al., 2004). Both of these observations lead to the question of whether these Wnt genes form a functional hierarchy during MHB development. In this hierarchy we postulated *wnt3a* had a superior role in MHB development based on previous studies and when we inhibit *wnt3* and *wnt3a* the MHB is absent. We also postulate based on our observations, *wnt1*, *wnt10b* and *wnt3* have inferior roles in MHB development. We observed *wnt1* in the MHB is absent when *wnt3* and *wnt3a* are inhibited. *wnt3* is absent in the MHB in *wnt3a* injected Df^{w5} embryos and *wnt3a* is absent in the MHB in *wnt3* injected Df^{w5} embryos. These data suggest with the different combination of inhibition of Wnt genes, Wnts form a hierarchy during MHB development. *wnt3a* appears to play a more superior role in regulating MHB development while *wnt3*, *wnt1* and *wnt10b* play a more inferior role in MHB formation.

fgf8a and pax2a regulate wnt genes during MHB development

Studies show that *fgf8a* and *pax2a* are required for MHB patterning and development. In both *fgf8a (x15)* and *pax2a (noi)* mutants, *wnt1* is activated but its expression is lost during somite stages (Lun and Brand, 1998; Reifers et al., 1998). We observed *wnt3, wnt3a, wnt10b* are absent in the MHB in *noi* and *x15* mutants at somite

stages. Studies show *pax2a, fgf8a*, and *wnt1* form a regulatory loop during somite stages in MHB formation (Raible and Brand, 2004). Our data suggest *pax2a* and *fgf8a* regulate Wnt expression in the MHB during somitogenesis.

Our results suggest there is a differential requirement for Wnt genes for MHB development during somitogenesis. *wnt3a*, *wnt1*, and *wnt10b* appear to be required for MHB development during somitogenesis but not *wnt3*. At 24 hpf, Wnt genes act in combination during MHD development but their requirements in this process are different. Wnt genes form a hierarchy to regulate MHB formation with *wnt3a* at the top. Also *pax2a* and *fgf8a* regulate Wnt genes expression during somitogenesis. Our data suggest Wnt genes have differential requirements in MHD development and more studies need to be performed to better understand their specific roles in MHD formation.

CHAPTER V

SUMMARY

Conclusions

I have examined the role of Wnt signaling in the temporal patterning of the MHD. I first asked if there is a relationship between the timing of Wnt signaling and the process of anterior posterior patterning. I inhibited Wnt signaling at multiple developmental time intervals and assayed Wnt signaling activity and anterior posterior neural patterning. My results show that at early stages in development, Wnt signaling is needed for global patterning of the anterior posterior neural axis. Subsequently, between 9.5 and 12 hpf, Wnt signaling is required for MHB development, and between 14 and 16 hpf, Wnt signaling is needed to maintain the MHB gene regulatory network. These data suggest that Wnt signaling is required during discrete time windows for different functions of anterior posterior patterning.

I also asked if there is a relationship between the process of neural posteriorization and the induction and patterning of the MHD. To answer this question, I inhibited Wnt signaling at 7 hpf and examined the progression of phenotypes by performing in situ hybridizations using forebrain, midbrain, MHB and hindbrain markers. I found that Wnt signaling is needed to activate *wnt1* and *eng2a*. I demonstrated that inhibition of gastrula Wnt signaling causes an expansion of forebrain fates into the posterior and a reduction of midbrain and MHB. These data suggest 7 hpf Wnt signaling is required to repress forebrain fates while promoting MHB and midbrain fates between 11.5 and 16 hpf.

There are four Wnt genes, *wnt1*, *wnt10b*, *wnt3*, and *wnt3a*, with overlapping yet distinct expression patterns in the MHD. Studies show *wnt1*, *wnt3a*, and *wnt10b* work cooperatively in MHB formation (Buckles et al., 2004). *wnt3a*, *wnt1*, and *wnt10b* are activated at 8-9 hpf, while *wnt3* is not activated until 11.5 hpf (Lekven et al., 2001; Lekven et al., 2002; Clements et al., 2009). This led me to ask whether the functional role of *wnt3* is separate from *wnt3a*, *wnt1*, and *wnt10b*. I observed that *wnt1*, *wnt10b*, *wnt3*, and *wnt3a* work in combination to regulate MHD patterning, but have differential requirements. I observed that *fgf8a* and *pax2a* are needed for regulation of the different Wnt genes during somite stages. These data suggest *wnt3a* is required during somite stages for specification of the MHD, while *wnt3* in combination with *wnt1* and *wnt10b* is needed for development of the morphological MHB constriction.

These studies help us to better understand how timing of Wnt signaling plays a role in the spatial patterning and cell fate specification of MHD development. These experiments also give a better understanding of how different Wnt genes interact in MHB formation. Understanding how the MHB, an organizer of midbrain and hindbrain patterning, develops will help us to better understand how neural induction and neural posteriorization occur. This is important in gaining insight into how the central nervous system is regionalized along its anterior posterior axis.

An interesting point that has not been addressed is why Wnt signaling is not required during gastrula stages for MHB development, despite numerous studies showing multiple roles for Wnt signaling in neural anteroposterior patterning, and also showing a direct role for Wnt signaling on the neural plate to position the MHB (Rhinn

et al., 2005). One possibility is that Wnt signaling regulates anterior posterior patterning of the neural plate, but is not specifically required for the induction of posterior neural fates, including MHB. To examine this possibility, we could inhibit Wnt signaling at 3 hpf and fix embryos beginning and ending at gastrulation and perform in situ hybridizations using *otx2* and *gbx1*, which position the MHB, *eng2a*, *pax2a*, and *wnt1* which are expressed in the MHD and regulate MHB development, and *zic1* and *gbx2* which are expressed in the forebrain and anterior hindbrain respectively. These experiments would allow us to examine if positioning of the MHB or the regulation of the MHB GRN are dependent upon Wnt signaling. Another point is the immediate absence of *eng2a* when Wnt signaling is inhibited during late gastrulation, suggesting Wnt signaling activates *eng2a*. Does Wnt signaling directly or indirectly activate *eng2a*? To answer this question, we could place the *eng2a* coding sequence under the control of a *wnt1* promoter and inject this construct into HSdkk-GFP/+ embryos. We could the perform a heat shock at 7 hpf and examine embryos at 10.5 hpf and perform in situ hybridizations using *eng2a* to observe if its expression returns. Further experiments should be performed to examine the differential requirements of Wnt genes in MHB formation.

Our data suggest Wnt genes have different requirements during MHB development. Is wnt3 activated later in development to prevent cell death or increase cell proliferation in combination with *wnt1* and *wnt10b*? To examine this possibility, we can knock down *wnt3* with morpholinos and perform a TUNNEL assay to examine if

apoptosis is affected and phospho-histone H3 staining to examine if there is an increase in cell proliferation.

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