TESTING THE ROLE OF WNT8A DIFFUSION IN WNT MEDIATED NEURAL POSTERIORIZATION

A Thesis

by

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ABSTRACT

This study assesses the role of diffusion of Wnt8a in patterning the anterior posterior (AP) axis of the neural plate. The vertebrate neural plate is subdivided into different morphological domains that define different functional regions. Failure to establish these morphological domains in the correct size have been shown to be associated with neuropathologies such as schizophrenia. While the adult brain is highly complex morphologically it begins as a simple sheet of cells. The neural ectoderm is first established on the dorsal side of the embryo, being activated from non-neural ectoderm by the presence of low levels of Bmp signaling. The neural plate initially lacks any AP pattern and will take on a forebrain fate if no additional signals are received. One of the key genes in establishing early posterior neural fate is *wnt8a*.

wnt8a is expressed in the margin of the embryo and has been shown to posteriorize the neural plate in a dose dependent manner, with higher levels of Wnt signaling inducing a more posterior fate. Wnts are lipoproteins that are secreted. However, their hydrophobic nature due to their lipidation has raised the question of how Wnt8a is able to diffuse across the neural plate to establish a posteriorizing gradient. Recent experiments in *Drosophila Melanogaster* have suggested that membrane tethered variants of *wg*, the Wnt homolog, can still pattern the wing disc. Our research was focused on testing if diffusion was necessary in vertebrate neural patterning.

We generated two transgenic lines, Tg(*otx2*FM*:h2bcitrine)* and Tg(*en2aDRE:egfp)* to allow imaging of the developing neural plate during epiboly. Neither of these transgenes were expressed at sufficient levels during early neural patterning, making them inappropriate for this study. However, the expression pattern of Tg(*en2a*DRE*:egfp)* suggests a novel positive feedback loop in the regulation of *en2a*. Testing of the Wnt8a diffusion hypothesis using a membrane tethered variant of *wnt8a* did not posteriorize the neural plate, but instead affected cell/cell cohesion and trunk development, an effect that was not predicted. While our research did not answer the question of the necessity of Wnt8a diffusion we did expand our knowledge of the regulation of neural patterning genes.

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CONTRIBUTORS AND FUNDING SOURCES

This work was supervised by a thesis committee consisting of Professor Arne Lekven and Professor Bruce Riley of the Department of Biology and Professor Alvin Yeh of the Department of Biomedical Engineering.

Images taken of the Tg(*en2a*DRE*:egfp)* seen in Fig 6a ii,iii,iv as well as 6b and graphing of fluorescence across the neural tube in 6c was performed by Dr. Holly Gibbs of the Yeh Lab. All other work conducted for the thesis was completed by the student independently.

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CHAPTER I

INTRODUCTION

The vertebrate brain is divided into a series of morphological domains that separate different functional regions of the brain. Each domain houses groups of neurons that serve specific roles in the behavior and function of the organism. Alterations to the size or shape of one of these domains during development can have far reaching repercussions. One region of interest is the cerebellum, as changes in the size or shape of the cerebellum have been associated with several neuropathologies including autism and schizophrenia (Andreasen and Pierson, 2008; Piven et al., 1997). While these correlation studies are possible using MRI and similar techniques in humans, the base cause of these neuropathologies will only be understood by looking at the development of embryos of model organisms.

Zebrafish as a model organism to study neuropathologies

Zebrafish are a small fresh water fish found in India (Engeszer et al., 2007). They have several features that make them an ideal model for studying neural development. Zebrafish develop externally, allowing easy collection and observation of development compared to mammalian models (Westerfield, 2000). In addition, the embryos are

transparent during early development, further easing imaging of the developing neural tissue (Kimmel et al., 1995). Because of these reasons, zebrafish can serve as an excellent model to better understand the mechanisms behind development of different regions of the brain, including the cerebellum. This will allow us to better understand the mechanisms causing changes to the size and shape of domains of the brain that cause neuropathologies.

History of vertebrate neural development

Embryogenesis is the transformation of an organism from a single cell into a specialized multicellular creature. Early research into embryogenesis utilized transplantations of tissues of different locations and stages of development to better understand the mechanisms that drive embryogenesis. Transplantation of the dorsal lip of an amphibian embryo prior to gastrulation to the ventral side of another embryo led to the formation of two fused embryos, resulting in the conclusion that the dorsal lip of the embryo was able to "organize" the tissue around it (Spemann and Mangold, 2003). This was intriguing because when any other tissue at this time point was transplanted it would instead take on the fate of the surrounding host tissue (Spemann and Mangold, 2003)These findings introduced the idea that specific tissues are capable of changing the fate of cells around them to drive the development of highly complex structures.

Transplantation experiments continued to provide invaluable insight into embryonic development, including the neural plate. It was shown that the neural plate forms by first activating a region of ectodermal tissue to become neural ectoderm, followed by a transformation phase where the neural ectoderm, which initially is fated to become anterior neural tissue, is posteriorized to establish the AP axis (Nieuwkoop, 1952, Fig 1.). While these transplantation experiments led to major breakthroughs in understanding embryonic development, the field was still missing a major piece of the puzzle: the identity of the signals that cause cell fate changes to occur. It was proposed that patterns could be established along the embryonic axes through secreted chemicals that diffuse from a source and produce a concentration gradient. Pattern is established when the tissue of the embryo responds to these different concentrations of chemicals by taking on different cellular fates (Wolpert, 1968). Secreted proteins could serve as the secreted signal that generated these patterns, but additional work would be required before the genes could be identified.

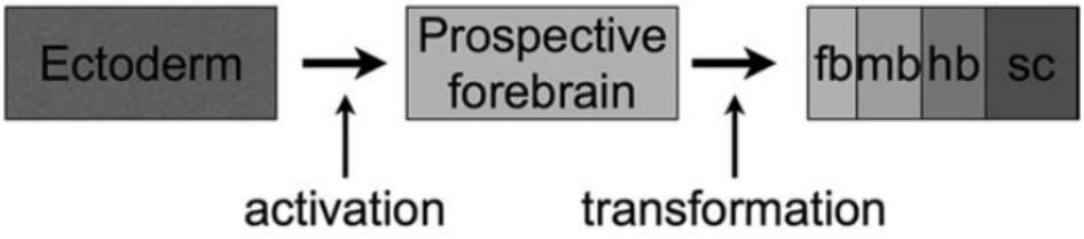

Figure 1. Historical model of early patterning. Adapted from: Green, D., *et al.* "Vertebrate Nervous System Posteriorization: Grading the Function of Wnt Signaling. *Developmental Dynamics*. 244(3), 507-512.

Before delving into the signaling events that pattern the neural plate, it is important to understand the morphological changes that occur in the embryo during the process of neural plate patterning. As this work focuses on the use of zebrafish as a model, we will focus on the morphological events of the zebrafish (CNS). From a single cell, the zebrafish begins development by undergoing a rapid series of cell divisions until four hours post fertilization (hpf), where the mass of cells, known as the blastoderm, migrate from the animal pole to the vegetal pole to cover the yolk, an event called epiboly (Kimmel et al., 1995). It is during epiboly that the neural ectoderm is first activated (Kimmel et al., 1995). The neural ectoderm begins as a simple epithelial sheet, the tissue then begins formation of a three-dimensional structure, the neural keel, shortly after epiboly is complete (Kimmel et al., 1995).The neural ectoderm forms a cylindrically shaped solid neural rod, a structure that persists from 11hpf to 16hpf Finally, fluid filled ventricles form in the center of the neural rod, after which the CNS is referred to as the neural tube (Kimmel et al., 1995). For these morphological events to form a brain with the correct dimensions requires the proper regulation of signaling events across the neural ectoderm that establish the pattern of fates along the anteroposterior axis.

Signaling events that pattern the AP axis of the neural plate

Neural plate development initiates when neural ectoderm is induced from dorsal ectoderm (Nieuwkoop, 1991). This is induced by the inhibition of Bmp signaling, which

is highest on the ventral side of the embryo (Baker et al., 1999). To establish the neural ectoderm dorsally, Bmp inhibitors including Noggin, Chordin and Follistatin are secreted from the organizer in the dorsal lip to establish a region of low Bmp signaling (Lamb et al., 1993; Piccolo et al., 1996; Zimmerman et al., 1996, Fig 2.). When the neural ectoderm is first induced, it is fated to become only anterior neural tissue in the absence of secondary signals that establish anteroposterior polarity.

To establish posterior neural fates including the midbrain, hindbrain and spinal cord a secreted posteriorizing morphogen is required. Wnt signaling has been identified as a critical secondary signal for neural posterior fate. It has been shown that Wnt signaling is essential to establishing posterior neural fates in vertebrates (Glinka et al., 1997; Sokol et al., 1990). In zebrafish, *wnt8a* is expressed in the embryonic margin (Lekven et al., 2001 ,Fig 2.). Loss of function of *wnt8a* leads to a loss of posterior structures in the neural ectoderm (Lekven et al., 2001), consistent with its requirement to specify posterior neural territories. Wnt signaling has been shown to posteriorize the neural plate in a dose dependent manner, with higher levels of Wnt signaling inducing a more posterior fate (Kiecker, 2001; Nordström et al., 2002). *wnt8a* posteriorizes the neural plate through its interactions with or regulation of transcription factors that segment the neural plate into different domains. *wnt8a* represses the expression of the anterior neural marker *otx2* and expands the posterior neural marker *gbx1* (*gbx2* in mice) (Millet et al., 1999; Rhinn et al., 2005). This is a very important step in establishing the AP axis, as the interface between *otx2* and *gbx1* are essential for positioning a secondary organizer at the

midbrain hindbrain boundary (MHB) (Joyner et al., 2000). The posterior region of this interface is dynamic in zebrafish, as this region transitions from expressing *gbx1* to expressing *gbx2* at the end of gastrulation (Kikuta et al., 2003). The transcriptional regulation of these genes is complex. The *otx2* locus is regulated through six enhancers driving expression in the neural plate (Kurokawa et al., 2004; Kurokawa et al., 2014). Specific enhancers of *gbx1* have not been identified. While the major players of establishing the secondary organizer in the MHB have been identified, how the neural plate transduces these signals is not well understood.

The positioning of the midbrain hindbrain domain (MHB) is important to later neural patterning because of its role as a secondary organizer. The MHB is positioned by the interaction between *otx2* and *gbx1*, which is nested within a broader neural plate domain, referred to as the MHB, that expresses several genes including *pax2, wnt1 and fgf8* which all have critical roles in patterning the region spanning the midbrain and cerebellum (Lekven et al., 2003; Lun and Brand, 1998; Reifers et al., 1998). Early patterning regulated through *wnt8a* is critical for the proper positioning of the MHB, and potentially the MH, and therefore has a large impact on the ultimate pattern of the AP axis of the brain.

While *wnt8a* is the earliest expressed Wnt gene involved in neural posteriorization, it is not the last, beginning a series of gene expression patterns that are critical for proper CNS patterning. As the midbrain hindbrain boundary is established, it begins activation

of multiple additional morphogens including *wnt10b* and *wnt1* which continue to refine the anterior posterior pattern of the embryo (Lekven et al., 2003) Other families of morphogens such as Fgf are also expressed. In cultured animal cap experiments it has been shown that Wnt signaling requires Fgf to posteriorize the neural plate(Nordström et al., 2002). Additionally, retinoic acid is involved in further patterning the hindbrain and is part of this larger regulatory network (Marshall et al., 1992). Each of these signals have distinct functions and are essential for patterning the neural plate along the AP axis (Kudoh et al., 2002), but together these morphogens separate the neural plate into different discrete regions which will be further subdivided to form the mature CNS. To understand the intricate signaling interactions that pattern the CNS, it is therefore essential to understand the mechanism of wnt8a mediated functions.

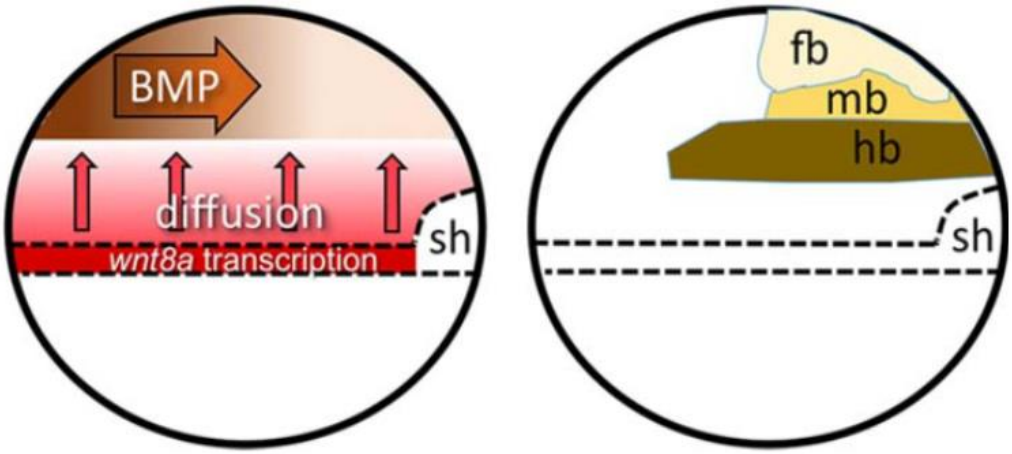

Figure 2. Summary of Wnt gradient model of neural posteriorization. Adapted from: Green, D., *et al.* "Vertebrate Nervous System Posteriorization: Grading the Function of Wnt Signaling. *Developmental Dynamics*. 244(3), 507-512.

The Mechanisms of Wnt signaling

Wnts are a family of secreted lipoproteins that have been associated with many roles in development, organogenesis and diseases (MacDonald, 2009). Initially discovered in *Drosophila,* wingless (*wg*) was shown to be essential for proper wing development (Cabrera et al., 1987). Wg is secreted and has been shown to diffuse over a gradient across the wing imaginal disc and patterns the AP axis of the wing (van den Heuvel et al., 1989). Homologs of *wg* were discovered in mammals by virtue of a mouse mammary tumor virus integration that caused cancer (Nusse and Varmus, 1982). Subsequent discoveries have cemented the fact that its role in embryonic patterning is conserved

across metazoans (Hobmayer et al., 2000). Wnts have been revealed to be a highly conserved and diverse family of morphogens involved in many aspects of embryonic development.

Wnts are secreted lipidated proteins (Willert et al., 2003) that signal through the co-receptors Lrp5/6 and Frizzled (Frzd) that coat Wnt responsive cells (Wodarz and Nusse, 1998, Fig 3.). The lipidation of Wnt8a is important, as it directly affects its ability to bind to the hydrophobic pocket of Frzd8 (Janda et al., 2012). Wnt binding to Frzd and Lrp5/6 induces the association and sequestration through vesicles of Gsk3-β(Vinyoles et al., 2014). The loss of cytoplasmic Gsk3-β leads to the destabilization of the APC destruction complex (Stamos and Weis, 2013, Fig 3.). Under non-signaling conditions, the APC destruction complex targets β-catenin for degradation in the proteosome (Stamos and Weis, 2013, Fig 3.). Upon the destabilization of the APC complex, β-catenin will then enter the nucleus and alter transcription (Wodarz and Nusse, 1998). At a Wnt responsive locus are a series of Tcf/Lef binding sites that in the absence of Wnt signaling are occupied by Tcf/Lef bound to the co-repressor Groucho (Wodarz and Nusse, 1998). This generates a repressive environment and shuts off transcription of Wnt target genes (Wodarz and Nusse, 1998). In the presence of β-catenin, Groucho is out competed in its binding to Tcf/Lef, thereby derepressing the locus (Wodarz and Nusse, 1998). How Wnts travel from their secretion source to target cells has been a subject of some disagreement. Wg has been shown to be secreted and diffused across a gradient in the wing disc, and it has been hypothesized that this mechanism is conserved for Wnt

transportation (Zecca et al., 1996). Recent work has shown that Wg diffusion is not required for proper patterning of the wing disc (Alexandre et al., 2014). This raises into question whether diffusion of other Wnts is necessary for proper patterning. Unlike Wg, vertebrate Wnts are lipidated and are therefore hydrophobic in nature; pull-down experiments have shown that Wnt1 is mostly found in the cytoplasm and is associated with membranes and rarely in the extracellular matrix (Parkin et al., 1993). How then does Wnt delivery occur to establish an AP signaling gradient?

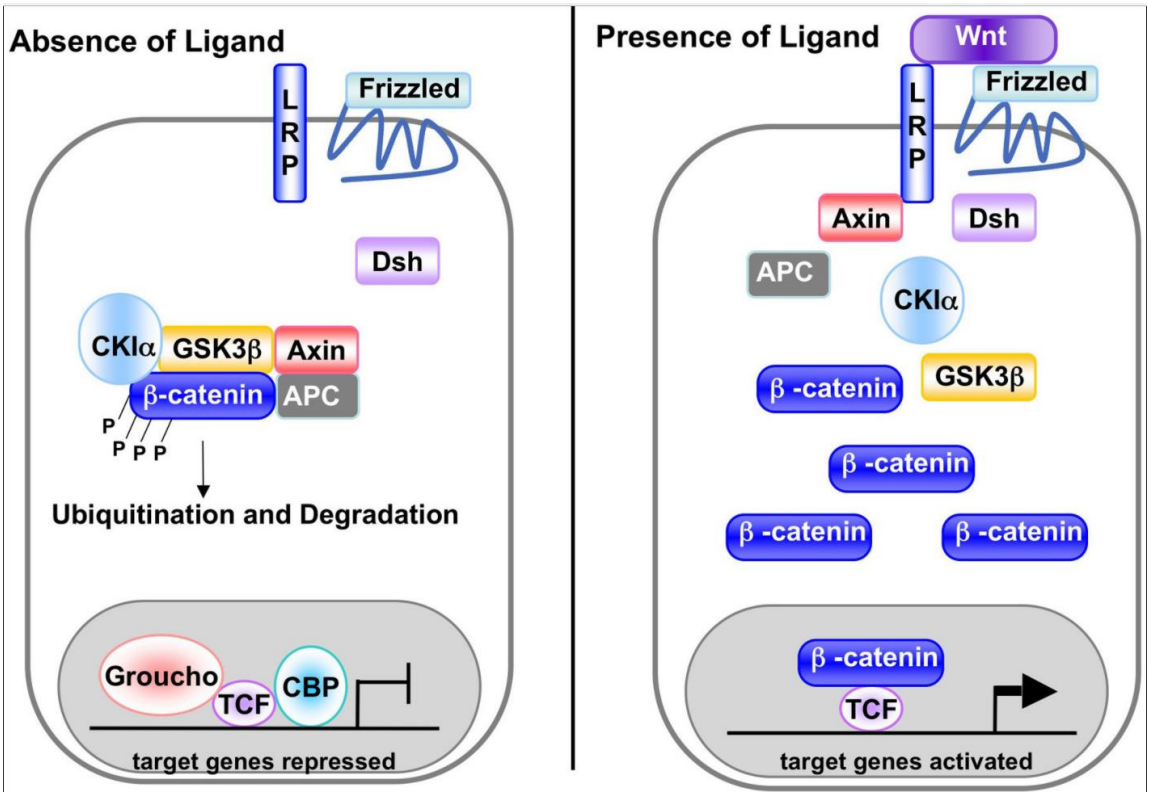

Figure 3. Summary of the canonical Wnt signaling pathway. Adapted from: Eisenmann, D.M, Wnt signaling (June 25, 2005), *Wormbook*, ed The *C. elegans* Research Community, Wormbook.

Filopodial transport and patterning

Cytonemes are small extensions emanating from cells that have the potential to extend multiple cell lengths away (Ramírez-Weber and Kornberg, 1999). The discovery of cytonemes was initially stymied by their very fragile construction, as they are destroyed by most fixation techniques (Ramírez-Weber and Kornberg, 1999). Cytonemes provide an important functional role in patterning *Drosophila,* and it has been shown that cytonemes coated with receptors can extend into Dpp diffusion ranges (Hsiung et al., 2005). This increases the range to which these morphogens can signal to the surrounding cellular environment. Similar extensions, named filopodia, have been identified in vertebrates as well. In chick limb buds, filopodial extensions coated in Shh as well as its receptor Ptch1 have been reported (Sanders et al., 2013). The Shh+ filopodia contact each other and allow Shh signaling to reach far across the limb bud (Kondrashov et al., 2011). Recently this phenomenon has been discovered in neural plate patterning, including the observation of filopodia coated with Wnt8a (Luz, 2013; Stanganello et al., 2014). The role of filopodia in Wnt signaling is particularly intriguing because it would explain how a hydrophobic protein is able to signal across a wide field of cells.

Goals

The focus of this study is to determine if diffusion of Wnt8a is required for AP patterning in the neural plate. The approach is to generate a membrane tethered fusion of

Wnt8a that will not be able to diffuse after secretion. The effect of this membrane tethered Wnt8a will be assayed using transgenic reporters that are expressed in different domains of the neural plate. The experimental design would determine if Wnt8a diffusion is required for neural patterning or if there is a secondary transportation mechanism.

We successfully generated two transgenic reporters: Tg(*otx2FM:h2bcitrine),* which is expressed in the midbrain, and Tg(*en2aDRE:egfp,)* which is expressed in the MHD. Neither of these reporter lines express detectable fluorescence during early neural patterning, even though reporter transcription is evident. While these lines were not useful for the goal of understanding Wnt8a diffusion, several novel elements of the transcriptional regulation of these genes was shown.

Several membrane tethered *wnt8a* constructs were generated and confirmed to be localized to the plasma membrane. However, these constructs failed to posteriorize the neural plate when overexpressed, instead leading to cell adhesion and trunk defects. This made it difficult to ascertain any direct effects of the membrane tethered Wnt8a.
Our study attempted to address the requirement of Wnt8a diffusion in vertebrates, but unforeseen phenotypes associated with attempting to tether Wnt8a to the membrane has made addressing this question difficult. However, the two transcriptional reporters generated for this project could be useful tools for future experiments designed to better understand neural development in vertebrates.

CHAPTER II

GENERATION OF TRANSGENIC REPORTERS TO MEASURE NEURAL AP PATTERNING

The goal of this portion of the project was to design a series of transgenic markers that would allow us to visualize the effect of Wnt signaling on neural plate patterning. To assist with this, we selected genes that were expressed in spatially discrete areas along the AP axis at 75% epiboly. Candidate enhancers were identified, cloned and described below.

Materials and methods

Reporter plasmid and vector construction

The *otx2*FM enhancer construct was assembled according to a previous publication (Kurokawa et al., 2004), in a lab Tol2 plasmid that expresses EGFP. The *en2a*DRE enhancers were cloned out of plasmids generously provided by the Ingham lab (Kurokawa et al., 2004; Maurya et al., 2011). *otx2*FM was successfully ligated upstream of *cfos* minimal promoter of the Tol2 construct pT2AW1. e*n2a*DRE, which contains the 1.5kb *en2a* minimal promoter, was successfully ligated downstream of a Kozak sequence. Nuclear localized fluorophores were amplified from pCS2P+*h2bcitrine*

(addgene #53572) and pCS2P+*egfp* (addgene #53744) downstream of the *otx2*FM and *en2a*DRE, respectively. Successful cloning was confirmed through Sanger Sequencing. All primers used in construction of these plasmids can be found in Table 1

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Zebrafish husbandry

Zebrafish are cared for following standard protocols(Westerfield, 2000). All wildtype strains are TL/AB hybrids.

Generating stable lines of reporter plasmids

Single cell WT embryos were injected with either 50pg pT2AW1*otx2*FM:*egfp* or pT2AW1*eng2a*DRE:*egfp* mRNA along with 30pg *transposase* mRNA. Embryos were raised to 24hpf and screened for fluorescence. Embryos with fluorescence were raised. Transgenic zebrafish were outcrossed to WT fish and offspring were screened for fluorescence. Embryos displaying fluorescence were isolated and raised to establish stable transgenic lines.

Describing transgenic reporter lines

Transgenic embryos for Tg(*otx2FM:egfp*) and Tg(*en2aDRE:egfp*) were collected at various time points and live imaged using Zeiss Imager Z1 and Zen imaging software. Z

stack images were projected into a single image using Fiji (Schneider et al., 2012). Images were edited for contrast using Photoshop. I*n situ* hybridization was performed following standard protocols (Thisse and Thisse, 2008). Images were taken using an Olympus SZX12 and SPOT imaging software. Images were edited for contrast using photoshop. Time course imaging of *Tg(en2aDRE:egfp*) was performed using a point scanning 2-photon microscopy system in the Yeh lab (Gibbs et al., 2014).

en2aDRE enhancer analysis

The zebrafish *en2a*DRE sequence was compared to the human (hg19), mouse(mm9), *Xenopus*(xenTro2) and Lizard(annoCar2) genomes with BLAT. Further sequence conservation analysis was performed using EvoPrint (Odenwald et al., 2005). Transcription factor binding sites were identified in conserved regions using PROMO (Messeguer et al., 2002).

Primer Name	Primer Sequence
otx2FM Fwd	TGGGCCCGATCCGTGGAATTGCTCTG
otx2FM Rev	ATCGCTGCAAGGTGGGGGGGTAAAG
en2a*DRE F*	TGGGCCCCGATCTGGCCATCTAGAGCG
en2aDRE R	ATCTGAGATCTGCTTTCCACCTTCC
h2begfp F for *en2aDRE*	ATCTGTAGATCTCTCAAGGCCTCTCG
h2begfp F for *otx2FM*	ATCTGTCTGCAGCTCAAGGCCTCTCG
SV40 R	TATAATCGATGTGGTTTGTCCAAACTCATC

Table 1. Primers for construction of fluorescent transgenic neural markers.

Results and discussion

en2a:DRE enhancer is expressed in the MHD after 15ss

To analyze the effect of membrane tethered Wnt8a on the neural plate, we constructed fluorescent transgenes to mark neural plate domains. Reviewing the literature, we identified *en2a* as a promising candidate for this study. e*n2a* is an important transcription factor in midbrain hindbrain boundary development (Dworkin and Jane, 2013). It is expressed in the midbrain hindbrain domain at bud stage (Fjose et al., 1992). A cis-regulatory analysis of *en2a* identified several enhancers, including a downstream regulatory element (DRE) which is 1285 bp downstream of the *en2a* coding sequence (Maurya et al., 2011, Fig 4.). The previous study had reported that the DRE element was expressed in the neural tube, but did not include any data describing it (Maurya et al.,

2011). We received the construct and cloned it into a Tol2 transposable element backbone, pT2AW1, driving *egfp* expression (Fig 5.). After screening and isolating transgenic heterozygotes, we analyzed its expression patterning using fluorescent microscopy.

A.

B.

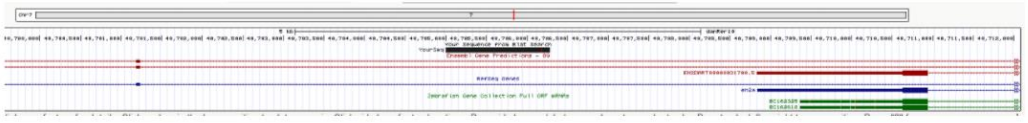

Figure 4. Sequence and location of *en2aDRE* in zebrafish. A) *en2aDRE* highlighted in blue upstream of 1.5kb *en2a* minimal promoter. B) UCSB genome browser showing *en2a* locus. Black box represents *en2aDRE*.

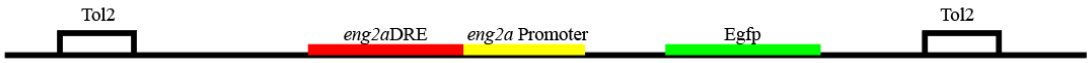

Figure 5. Cloning of *en2aDRE:egfp* reporter.

Expression of Tg(*en2a:DRE:egfp*) at 24hpf is localized in the MHD of the zebrafish neural tube, with higher levels of expression in the midbrain side of the domain (Fig 6a). Expression can also be seen along the trunk of the animal localized to muscle pioneer cells (Fig 6a). To determine if the transgene was expressed at earlier time points we performed a time course from 75% epiboly until 24hpf. Expression of the transgene was first observed at the 18 somite stage (ss), much later than we had anticipated (Fig 6b). At 18ss, expression was uniform across the midbrain hindbrain domain, but decreases in the cerebellum leading to an apparent gradient of expression (Fig 6b,c).

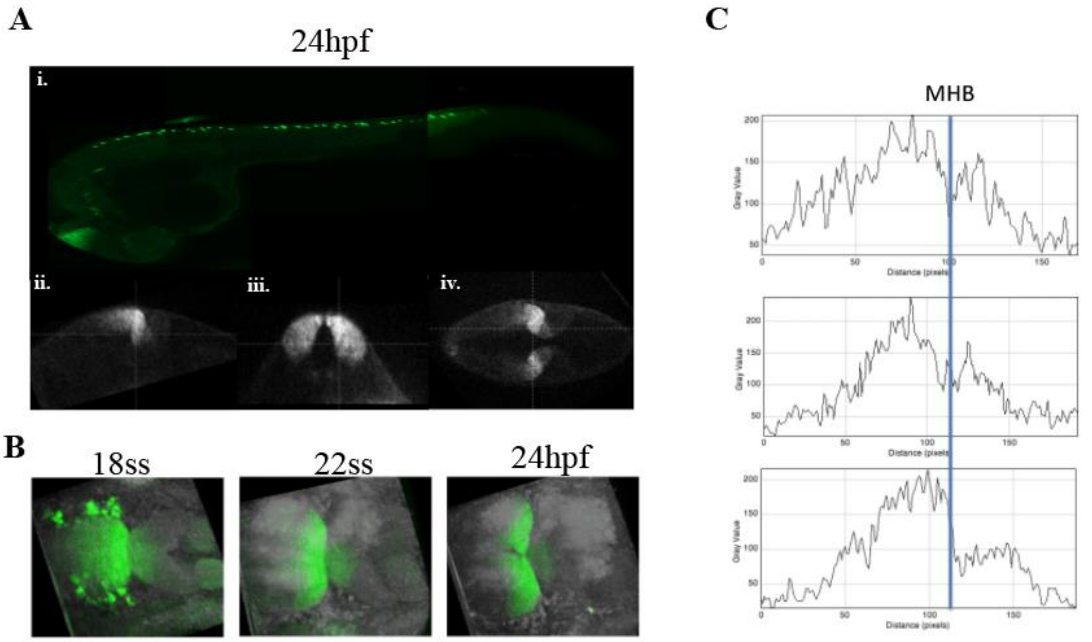

Figure 6. Expression pattern of Tg(*en2aDRE:h2begpf)*. A) i. lateral view anterior left. ii. Lateral view anterior left. iii. Transverse view, anterior forward, dorsal up. Iv. Dorsal view anterior left. B) Dorsal view anterior left.

As our primary goal was to generate a MH marker that would express when *wnt8a* is patterning the neural plate, the late expression pattern meant that this line shows expression after the developmental time of interest and thus would not be able to assist us in better understanding the role of Wnt8a diffusion in patterning the neural plate. However, the expression pattern of the DRE at this later stage leads to the question of what the function is of the DRE in regulating *en2a* in the developing embryo.

Analysis of Tg(en2aDRE:egfp) reveals conserved repeated engrailed binding sites

To ascertain why the DRE drives expression at later time points than expected for an *en2a* enhancer, we analyzed the DRE element in detail. Our first step was to compare the DRE sequence to the corresponding element from several other animals including *Drosophila*, mouse, humans, *Xenopus*, and lizard. BLAT comparison of the enhancer in different organisms revealed partially conserved regions, though the position of the DRE relative to the *en2a* coding sequence varied significantly, with zebrafish being the closest and humans being the furthest away (Fig 7.). The human DRE contains a large 3kb insertion splitting the conserved sequence (Fig 7). A conserved element of approximately 150 bp was identified within the DRE using EvoPrint (Fig 7.) This suggests that the DRE may have a conserved role in regulating *en2a* expression across the animal phyla. Within this conserved region was a smaller segment of the DRE that was largely conserved across the animal phyla, designated as the ultra-conserved region (Fig 7.). The considerable conservation of this non-coding sequence between different taxa that have been separated by extensive evolutionary time suggests that there is a functional role for this sequence. We hypothesized that if there were a functional role for this region, then there may be conserved transcription factor binding sites. Using PROMO, we analyzed the ultra-conserved region and identified five tandem repeated *engrailed* consensus binding site sequences (Fig 7.).

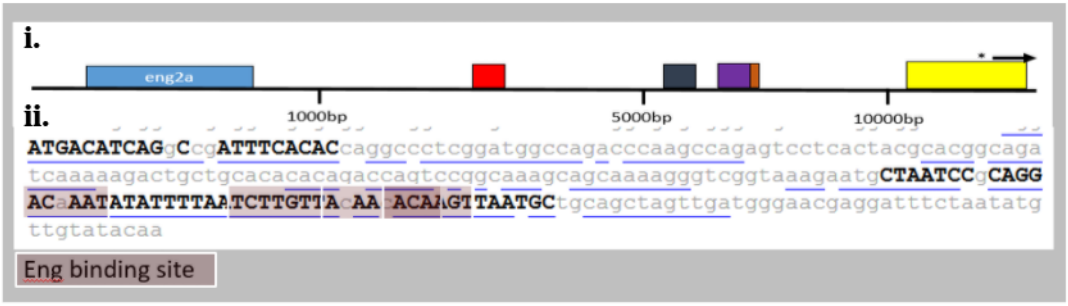

Figure 7. Conserved region of *en2aDRE*. Schematic of *en2a* locus. Zebrafish (red), *xenopus* (grey), mouse (purple), lizard (orange), human (yellow). *human *DRE* much larger due to 3kb insertion. Sequence shows conserved region. Blue underline sequence is conserved in at least two organisms, capitalized bold letters are conserved in all organisms.

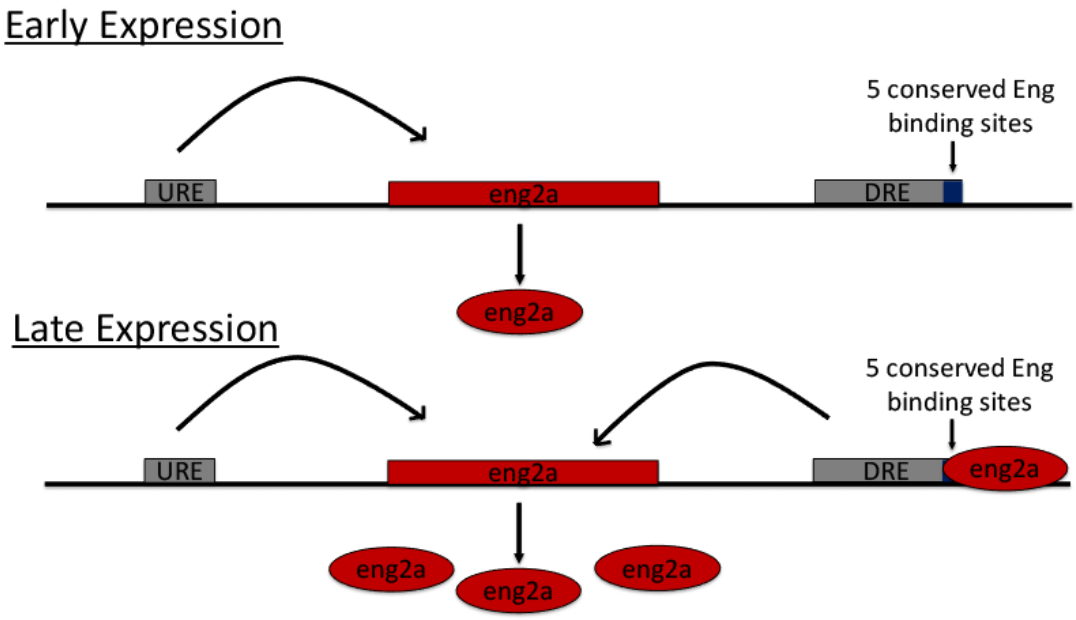

Figure 8. Schematic of DRE positive feedback loop hypothesis.

These findings suggest that the DRE may serve as a positive feedback loop, activating expression of the *en2a* promoter in the presence of En2a (Fig 8.). This would explain the late activation of Tg(*en2aDRE:egfp)*. However, why the expression of the DRE is in a

gradient across the MHD and why that gradient refines into the midbrain is unknown. Future experiments could analyze this by deleting elements of the DRE and testing those modified enhancers for expression. Of interest would be the deletion of the ultra-conserved element and specifically the deletion of the *engrailed* binding sites.

Tg(otx2FM:h2bcitrine) enhancer replicates endogenous expression of otx2 in the neural plate

otx2 is expressed in the anterior neural plate from 60% epiboly until 5ss, at which point expression is lost in the forebrain and expression is limited to the midbrain (Li et al., 1994). This makes it an ideal candidate to mark anterior neural structures during early *wnt8a* patterning. Several enhancers of *otx2* have been previously identified, including the FM enhancer, which was shown to be strongly expressed in the midbrain at 24hpf (Kurokawa et al., 2004). The *otx2*FM enhancer is composed of two elements a 612 bp sequence that is located approximately 21 kb upstream of the *otx2* locus (Fig 9 and 10). The second element is a 1137 bp region cloned directly upstream of the *otx2* start codon(Fig 9 and 10). Due to its expression in the midbrain, this enhancer was a strong candidate for being an effective reporter to analyze the effect of Wnt8a on neural patterning.

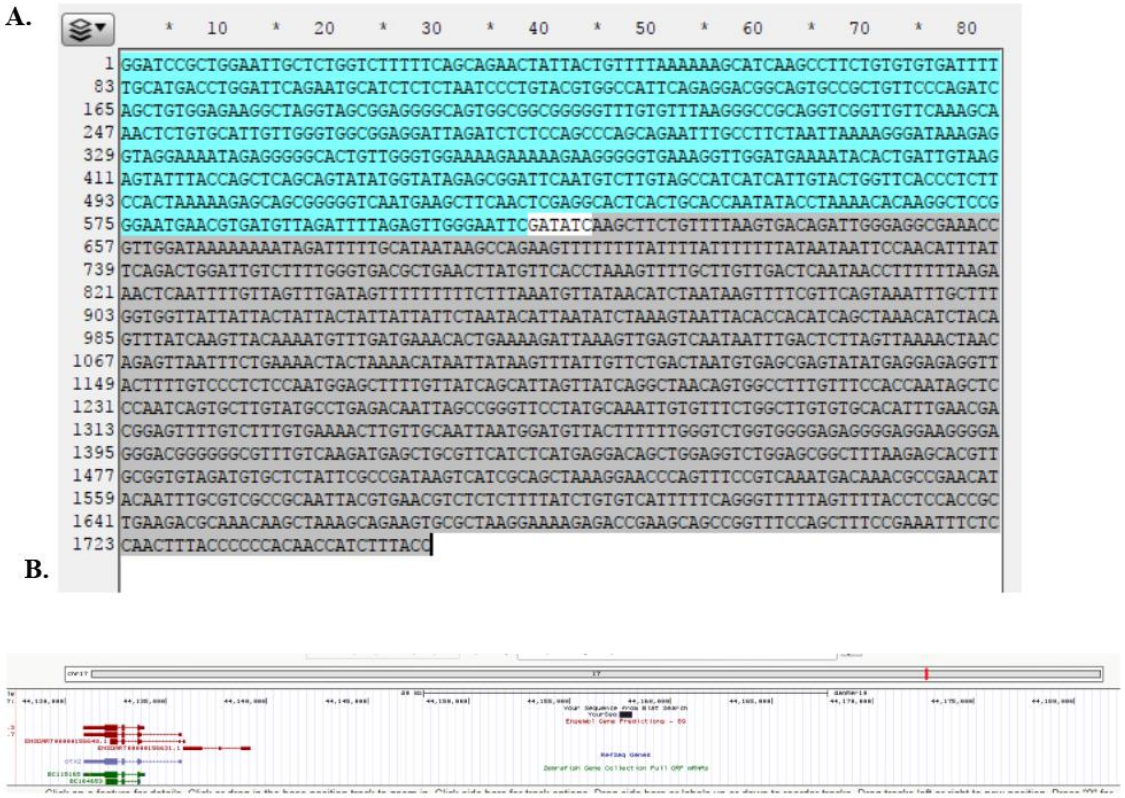

Figure 9. Sequence and location of *otx2*FM enhancer. A) *otx2*FM shown in light blue, grey highlight represents *otx2* promoter. B) *otx2FM* sequence mapped onto GRCz10 genome using UCSB genome browser. Black box represents *otx2*FM.

The *otx2*:FM enhancer was cloned into a Tol2 transposable construct driving *h2bcitrine* (Fig 10.). Transgenic lines containing multiple insertions of the enhancer were identified and utilized for all experiments. Fluorescent imaging of the *Tg(otx2FM:h2bcitrine)* showed expression first appearing at 90% epiboly in the yolk syncytial layer (YSL); however, no fluorescence was detected in the neural plate at these time points (Fig 11.). Fluorescence was first detected in the neural plate at bud stage, where it was expressed in the anterior region of the neural ectoderm (Fig 11.).

Expression in the forebrain region of the brain perdured until 10ss, where brightness levels decreased significantly in respect to midbrain expression (Fig 11). Expression of Tg(*otx2FM:h2bcitrine)* in the midbrain is visible until at least 30hpf (Fig 11.). *in situ* hybridization was performed at 75% epiboly, when endogenous *otx2* is expressed in the anterior neural plate. Expression of h2b-citrine was detected in the anterior neural plate (Fig 11.). Thus, these data suggest that the *otx2*FM enhancer successfully recapitulates *otx2* expression, even at epiboly stages, unlike Tg(*en2aDRE:egfp)*. However, likely due to slow folding and accumulation of H2b Citrine, its levels remain too low to detect fluorescence until bud stage.

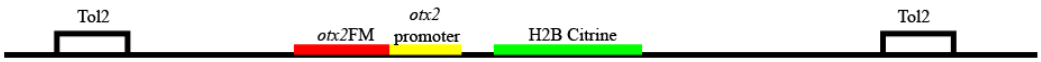

Figure 10. Schematic of T2AW1(*otx2FM:h2bcitrine)* reporter.

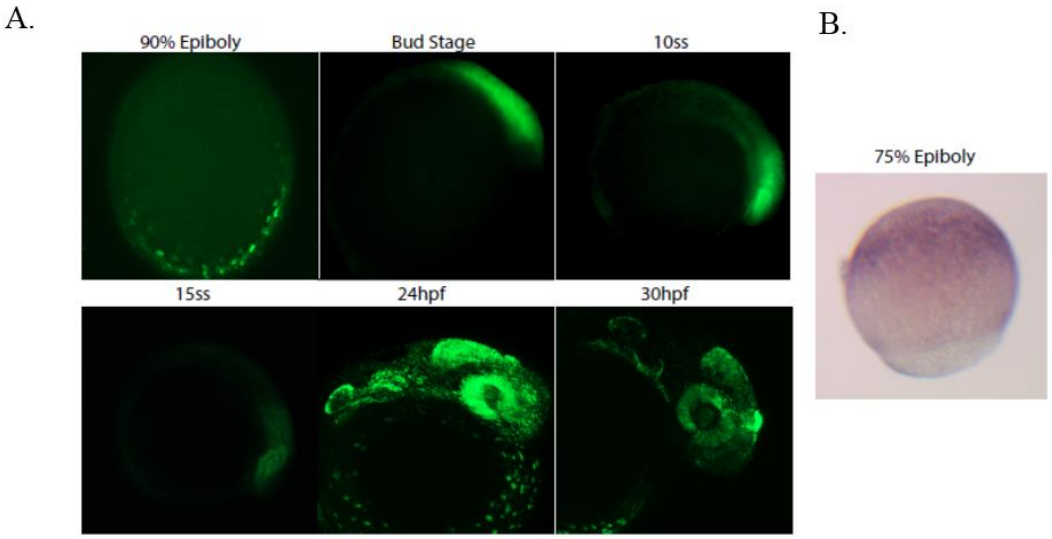

Figure 11. Expression pattern of Tg(*otx2FM:h2bcitrine)*. A) lateral view, anterior to the right. B) Dorsal view animal pole up.

Summary

The purpose of generating neural transgenic markers was to allow tracking of cells during and shortly after epiboly, where *wnt8a* is posteriorizing the neural plate. The expression pattern of Tg(*en2aDRE:h2begfp)* and Tg(*otx2FM:h2bcitrine)* are both detectable through fluorescence too late in development to be used for this purpose. However, by generating Tg(*en2aDRE:h2begfp)* we have learned about a previously unreported aspect of the DRE enhancer. The late expression pattern and gradient across the MHD makes the DRE an interesting element of *en2a* regulation. Our analysis into the enhancer revealed a possible positive feedback loop that should be investigated in future projects.

CHAPTER III

THE EFFECT OF OVEREXPRESSION OF MEMBRANE TETHERED WNT8A ON EMBRYONIC PATTERNING

The goal of this portion of our project was to test whether free diffusion of Wnt8a across the neural plate is necessary for proper anterior posterior patterning. To test this, we generated a series of membrane tethered *wnt8a* fusions. These constructs were injected into zebrafish embryos to test their ability to posteriorize the neural plate.

Materials and methods

Reporter plasmid and vector construction

To better understand the role of diffusion in *wnt8a* mediated neural posteriorization, a membrane tethered fusion of *wnt8a* was created. I used Expasy to identify the Wnt8a signal peptide (Gasteiger et al., 2003, Fig 12.). *wnt8a*ORF1 lacking the signal peptide (NSP) was ligated downstream of SP6 promoter in pCS2P+. The neurotactin transmembrane domain was amplified from the pCS2P+nrt plasmid obtained from the Jean-Paul Vincent lab (Alexandre et al., 2014). The *nrt* transmembrane domain was

cloned upstream of wnt8a in pCS2P+NSP*wnt8a*. All primers used in creation of these plasmids are in Table 2.

Primer Name	Primer Sequence	Restriction Enzyme Site Added
wnt8aORF1 NSP F	AGAGAGCTCGTGATGATGACAGGACC	Sac1
wnt8aORF1 R	TTACGTATCATCCACGACGCGTGTG	SnaB1
nrt Fwd	AGGATCCACCATGGGCGAACTCGAGG	BamH1
nrt Rev	GAATTCCGGCGGCAGGTCAAAGTCTCT	EcoR1
egfp Cla1 Nostop	TATAATCGATAGCTCGTCCATGC	Cla1
egfp Fwd	TATAGAATTCCTGGTCGAGCTGGACGGCGACG	EcoR1

Table 2. Primers for construction of membrane tethered *wnt8a*.

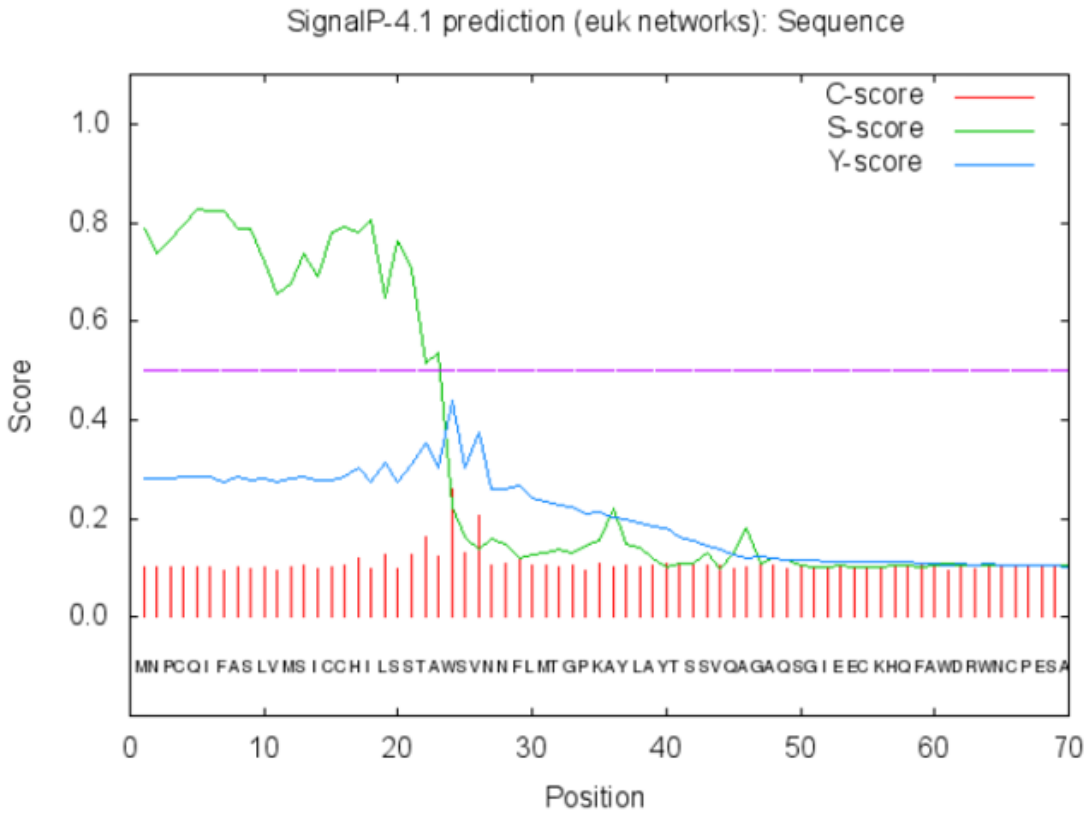

Figure 12. Prediction of *wnt8a*ORF1signal peptide using Expasy.

Membrane localization assay

Embryos were injected with 50pg of mem*wnt8a*ORF1, mem*gpf* or *egpf* mRNA at the 1 cell stage. Live embryos were imaged at dome stage using a Zeiss Imager Z1 and Zeiss Zen software.

Overexpression of membrane tethered Wnt8a

Embryos were injected with variable doses of mem*wnt8a*ORF1 or *wnt8a*ORF1 mRNA and grown to 24hpf. Live embryos were imaged with an Olympus SZX12 and SPOT imaging software.

Results and discussion

Design and production of membrane tethered wnt8a and controls

Due to the biscistronic nature of the *wnt8a* locus, there are two open reading frames (Lekven et al., 2001). We selected ORF1 to generate our membrane tethered fusions because this protein displays the broadest activity in functional assays (Lekven et al., 2001). To generate a membrane tethered fusion of *wnt8a* we fused the *neurotactin* (*nrt*) transmembrane domain to *wnt8a* ORF1. This construct is classified as mem*wnt8a*ORF1 (Fig 13.). The *nrt* transmembrane domain has been used in generating membrane

tethered *wg* fusions in Drosophila (Alexandre et al., 2014). To allow easy identification of the construct after injection, an *egfp* coding sequence was fused between the *nrt* transmembrane domain and *wnt8a* (Fig 13.). As this was a N-terminal fusion, the signal peptide normally found in the *wnt8a* ORF1 coding sequence had to be removed to stop cleavage of the Wnt8a protein from the transmembrane domain. As a control, an alteration of *wnt8a* was generated that lacked the signal peptide (no signal peptide, NSP) (Fig 13.). We predicted that the NSP*wnt8a* transgene should not have any effect on the embryo as it would not be able to be secreted from the cell. An additional control was designed that only contained the transmembrane domain fused to Egfp (NRT Control) (Fig 13.). All constructs were cloned into pCS2P+ to allow easy production of mRNA for injections.

Figure 13. Membrane tethered *wnt8a* and control constructs. Grey *nrt* transmembrane domain, black *wnt8a*ORF1, Blue *wnt8a*ORF1 signal peptide.

Testing membrane localization of tethered Wnt8a

Our first goal was to confirm that the memWnt8aORF1 fusion would properly localize to the plasma membrane. To do this, we injected zebrafish embryos either with mem*wnt8a*ORF1, with *pmemegfp* (a nuclear localized *egfp*), or with *egfp*. We hypothesized that if memWnt8aORF1 was localized to the membrane, then we would detect increased expression of Egfp in the membrane as well. Embryos were injected with 50pg of mRNA and visualized at dome stage to allow easy identification of individual cells. Fluorescence was localized to the membrane in both the mem*wnt8a*ORF1 and pmeme*gfp* injections confirming our hypothesis that the Nrt transmembrane domain was sufficient to localize the Wnt8a protein to the membrane of the cell (Fig 14.). Having confirmed that the construct properly localized to the membrane, we continued our experiments to determine if the construct acted in a similar manner to endogenous *wnt8a*.

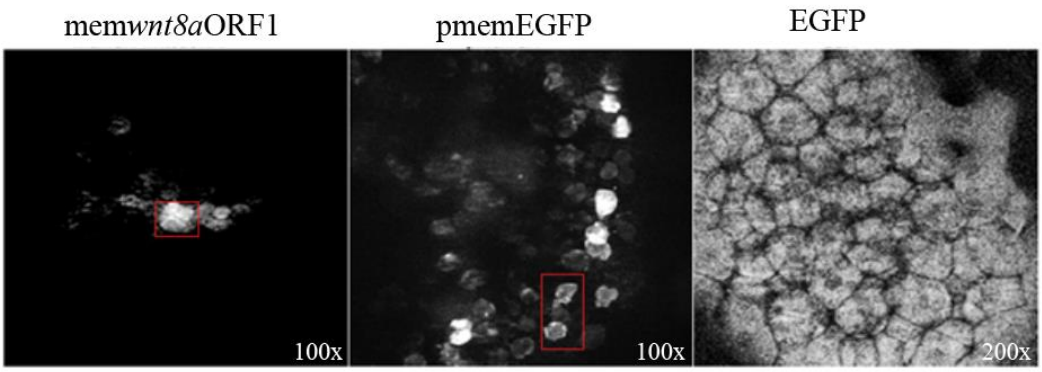

Figure 14. Localization of Memwnt8aORF1 to the membrane of cells at dome stage. Animal pole view.

Overexpression of memwnt8aORF1 leads to loss of cell-cell cohesion and trunk defects

To test our hypothesis that membrane tethered Wnt8a would be able to posteriorize the neural plate, we performed a dose-response experiment using mem*wnt8a*ORF1 as well as *wnt8a*ORF1. Embryos were injected at the 1-cell stage with varying doses of mRNA, and then were assayed for morphology at 24hpf. Variable phenotypes were observed in each injection and were classified according to severity. As expected overexpression of *wnt8a*ORF1 led to loss of anterior neural structures, most clearly seen by loss of the eye (Fig 15). However, overexpression of mem*wnt8a*ORF1 did not induce loss of anterior neural structures, instead leading to a range of novel phenotypes (Fig 15). Embryos failed to complete their developmental program either arresting prior to epiboly or gastrulation by 24hpf (Fig 15). Embryos that developed past epiboly had severe trunk defects, indicative of issues with epiboly (Fig 15). The occurrence of these phenotypes

correlated with the dosage of mem*wnt8a*ORF1(Table 3). This suggests that while the membrane tethered Wnt8a does not pattern the neural plate like endogenous Wnt8a it may have other effects on patterning. One possibility is that the alterations to the structure of Wnt8a induced by the transmembrane fusion may cause Wnt8a to interact with receptors that induce non-canonical Wnt signaling pathway. This pathway has been shown to be critical for proper convergent extension necessary for gastrulation (Yamanaka et al., 2002). If mem*wnt8a*ORF1 is affecting the non-canonical Wnt signaling pathway it would explain the gastrulation defects that we observed in the overexpression assay.

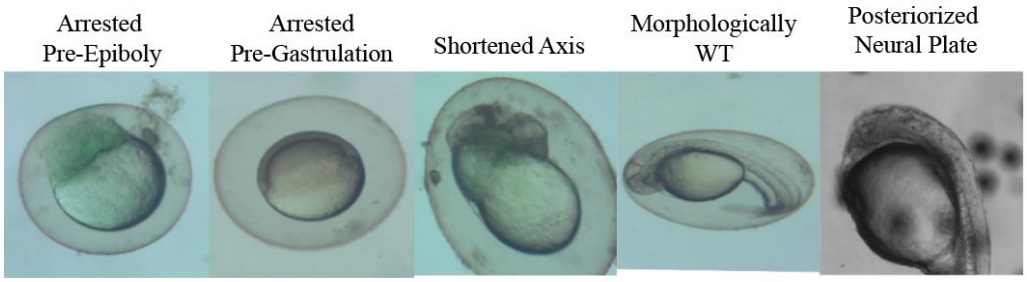

Figure 15. Classification of membrane tethered Wnt8a overexpression assay. All embryos grown at 29°C for 24 hours. Lateral view, anterior left.

	50pg mem*wnt8a* ORF1	100pg mem*wnt8a* ORF1	200pg mem*wnt8a* ORF1	400pg mem*wnt8a* ORF1	wnt8aORF1
Morphologically WT	20%	0%	28%	0%	0%
Shortened Body Axis	13%	33%	22%	0%	0%
Arrested Pre-Gastrulation	33%	25%	11%	7%	0%
Arrested Pre-Epiboly	33%	42%	39%	93%	0%
Loss of Eyes	0%	0%	0%	0%	100%
n	15	12	18	28	22

Table 3. Occurrence of different phenotypes for Memwnt8aORF1 overexpression assay.

Summary

We had hypothesized that a membrane tethered fusion of *wnt8a* would be able to posteriorize the neural plate in a dose dependent manner similar to endogenous *wnt8a*. However, we found that by fusing the *wnt8a*ORF1 to the *nrt* transmembrane domain at the N terminus, *wnt8a* is no longer able to posteriorize the neural plate but rather has a strong effect on the ability to progress through gastrulation. It is possible that this phenotype is specific to the *nrt* transmembrane domain, or to N-terminal fusions of *wnt8a*. To rule out these possiblities, future experiments should test other transmembrane domains located in different positions of the protein. A possible explanation for the novel phenotypes we see in the mem*wnt8a*ORF1 overexpression assay is that by changing the structure of Wnt8a, we have changed its binding to Frzd receptors. This could then lead to the ectopic activation of non canonical Wnt signaling pathways. Future experiments should test whether the mem*wnt8a*ORF1 construct is able to activate the non-canonical Wnt signaling pathway.

CHAPTER IV

CONCLUSION

The goal of our project was to test whether the diffusion of Wnt8a across the neural plate was required for proper AP patterning. We formed this hypothesis based on recent research in *Drosophila*, which showed that *wg*, the fly homolog of Wnt genes, could be replaced by a membrane tethered variant and wing patterning still progressed normally (Alexandre et al., 2014). To understand this phenomenon in a vertebrate model we attempted to create a similar assay in zebrafish.

To assist us in understanding Wnt mediated neural posteriorization we first attempted to generate novel toolsets to better map the neural plate in live embryos. To do this we identified two important AP patterning genes, *otx2* which is expressed in the anterior neural plate and *en2a* which is expressed across the MHD. We identified for both genes. From previous publications Upon generating our own series of transgenic lines using these enhancers to drive nuclear localized fluorophores we discovered that the expression pattern of these transgenes would not be appropriate for imaging neural plate development in live embryos around epiboly. Tg(*otx2FM:h2bcitrine)* was expressed in the neural plate at 75% epiboly as predicted, but levels were not sufficient to allow detection of the transgene in live embryos until bud stage. Tg(*en2aDRE:egfp)* was not

expressed until much later than we predicted and would not serve as a marker for early neural patterning. However, analysis of expression of Tg(*en2aDRE:egfp)* revealed that the enhancer had several interesting phenotypes, including a gradient of expression across the MHD. Additional experimentation should be done to continue to analyze the role of the DRE in regulating expression of *en2a* in the neural ectoderm.

To directly test the role of diffusion in *wnt8a* mediated neural AP patterning we generated membrane tethered fusion of *wnt8a*ORF1 and overexpressed it in zebrafish. We predicted that this construct should have a similar phenotype to the overexpression of untethered *wnt8a*ORF1, as its ubiquitous expression would allow *wnt8a* to signal across the neural plate regardless of diffusion. What we discovered though was that the membrane tethered variant of *wnt8a* had strong effects on cell/cell adhesion and trunk morphogenesis, but did not have a strong effect on neural plate posteriorization. We hypothesize that by fusing *wnt8a* to a transmembrane domain we may have changed the structure of the Wnt8a protein so that it activated non-canonical vs canonical Wnt signaling pathways.

In conclusion while our primary objective of understanding the requirement of diffusion for *wnt8a* mediated neural posteriorization was not successful we have learned several novel features about the regulation of neural patterning genes. In the future work identifying the necessary elements of the Tg(*en2aDRE:egfp)* enhancer could serve to better understand the regulation of *en2a* in the mid-somitogenesis brain.

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