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Anterior-Posterior Neural Axis Plasticity in the developing central nervous system of Xenopus laevis

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Anterior-Posterior Neural Axis Plasticity in the developing central nervous system of *Xenopus laevis*

A thesis submitted in partial fulfillment of the requirement for the degree of Bachelor of Science in Biology from the College of William and Mary

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ABSTRACT

Plasticity, the ability to redifferentiate and change cellular identity, is an integral part of development as cells must develop and specify while being able to respond to any changes in the environment. Many instances of cell identity changes have been identified during development, but the mechanisms that allow certain cells to do this while others remain in a fixed fate are unclear. The patterning of the neural axis in the central nervous system involves the specification and determination of neural cell identity along the anterior-posterior axis. While the mechanisms that determine the anterior-posterior neural axis early in development are well characterized, the plasticity that underlies this process remains poorly understood. While expression of genes such as *Suv4-20h, Prc2,* and *ezH2 are* known to bring about loss of potency-related factors, neither the temporal or spatial parameters of this developmental neural plasticity nor the molecular mechanisms governing the process are understood. This investigation was designed to examine the spatial-temporal limits of plasticity in the development of the vertebrate central nervous system. A rotation transplant system with a labeled donor embryo and unlabeled host embryo was used to transplant neural ectoderm covering the median half of the dorsal ectoderm from the donor embryo and rotate the anterior-posterior axis 180º onto the host embryo. This allowed for the testing of physically perturbing the axis and assaying its ability to re-differentiate. Results suggest that there is a period between mid gastrula (11.5) and late gastrula (12.5) during which the axis has been determined based on expression of regional marker genes along the axis (*XCG-1, Otx2, En2, and Krox20*) but remains competent to re-differentiation. In addition, the results indicate that the tissue itself loses competency as late gastrula embryos are still able to re-differentiate mid gastrula neural tissue to the correct pattern, but neural transplants from late gastrula embryos are unable to re-differentiate even in the presence of the mid gastrula inducing environment. Hence, the neural tissue remains plastic for a limited time period after acquisition of an anterior-posterior fate during development.

1. OVERVIEW OF THE PROBLEM

During development, cells exhibit extraordinary potential to differentiate into multiple cell types (Chambers et al., 2007; Lupo et al., 2014; Morgani and Brickman, 2014; Odorico et al., 2001). As the cells differentiate, there is a gradual restriction of cell fate as the cell becomes more determined in its fate (Estacio-Gómez and Díaz-Benjumea, 2014; Nicetto et al., 2013; Tanaka et al., 2014). Even though this restriction of cell potency occurs, cells are still able to respond to perturbations from their environment and be able to recover. The ability to regulate and alter cell identity in the face of changes to the environment is known as plasticity. Even in the adult nervous system cells are able to undergo changes in their physiology through receptor subtype switching (Kumar et al., 2002) and even neuronal to glial shifts in cellular identity (Malatesta et al., 2000).

This plasticity is strongly present in development as cells are being specified but have not yet become committed to their fate. While cells may receive signals to adopt a certain identity, the cells may not be fully determined to this identity until other developmental programs reinforce identity. Thus, while the cell is specified for its given fate, it may not be fully committed to that cell fate and be able to change its initial specification and adopt a different cellular fate. The labile nature of cells in development, even during nervous system patterning (Saha and Grainger, 1992), makes plasticity a central process in embryonic development. However, while factors that have induced changes in gene expression to lead to differentiation are fairly well understood, the plasticity

regarding the process is less well-studied (Skipper et al., 2010). The bounds and limits of this plasticity during cell-type specification are what this project has attempted to characterize.

2. REVIEW OF THE LITERATURE

2.1 Early Patterning: Fertilization and Early Cleavage

Early fate mapping experiments revealed that even during the 8-cell stage (blastula) the embryo already has undergone considerable patterning along its dorsal-ventral axis (Grant et al., 2013, 2014; Keller et al., 1992; Moody et al., 2013) and that certain cells have been specified to become dorsal. This initial identification of dorsal versus ventral is crucial to the determination of the nervous system as the dorsal ectoderm is what will give rise to the central nervous system. This patterning is specified during fertilization through maternal determinants and cytoskeletal rearrangements during fertilization. Initially, β-catenin is present throughout the oocyte prior to fertilization but is degraded by the presence of glycogen synthase kinase-3 (GSK-3) through activation of the destruction complex (Larabell et al., 1997; Liu et al., 2002; Miller and Moon, 1997). Presence of β-catenin leads to a dorsal fate (Funayama et al., 1995), while presence of GSK-3 leads to a ventral fate (He et al., 1995). Stabilization of β-catenin occurs through the expression of the Disheveled protein that acts to inhibit the activity of GSK-3 thus preventing the degradation of β-catenin (Metcalfe and Bienz, 2011). Disheveled is maternally expressed in the vegetal

cortex of the oocyte prior to fertilization. During the fertilization event, sperm binding triggers a cortical rotation and induces movement of the disheveled protein along microtubule arrays to the side opposite of sperm binding (Miller et al., 1999); this side gives rise to the dorsal portion of the embryo.

β-catenin acts as a transcription factor to upregulate the expression of genes such as *siamois*. The siamois protein will act as transcription factor in conjunction with vegetally expressed TGF-β proteins (*Vg1, VegT*) to lead to the expression of *goosecoid*, which is critical for the formation of the organizer (discussed below) (Brannon et al., 1997; De Robertis, 2009; Vonica and Gumbiner, 2007). These early events establish the parameters for gastrulation to occur by specifying regional identity.

2.2 Neural Induction and Pattern Formation: Gastrulation

Determination of dorsal endoderm during the blastula stages as described above induces the formation of Spemann's Organizer (De Robertis, 2009; Robertis, 2006; Vonica and Gumbiner, 2007). In the classic Spemann-Mangold experiments, the organizer of one embryo was transferred to the ventral side of another embryo. This induced the formation of a secondary axis on the host embryo. This revealed the importance of the organizer to induce cellular changes that lead to the formation of specific tissue types present on the dorsal side of the embryo such as the central nervous system (Keller et al., 1992; Robertis, 2006, 2009). Changes in cell shape on the dorsal endoderm where the cells at the edge of the organizer begin to elongate, forming bottle cells, are critical for the initiation of gastrulation. As these cells begin to lengthen, they start to invaginate

into the endoderm and cause other connected cells to invaginate with them. This invagination pulls the endoderm with the mesoderm inward to result in an embryo that has the ectoderm as the outer layer, the mesoderm as the middle layer, and the endoderm as the inner layer (Anand et al., 2015; Gilbert, 2000; Hardin and Keller, 1988; Keller, 1981). While these bottle cells are crucial for initiating gastrulation, they are not essential for its progress. Experimental analyses have revealed that removal of bottle cells during initiation of gastrulation prevents progression of gastrulation, but removal of the cells during gastrulation has no effect on the continuation of the cellular movements that were initiated (Hardin and Keller, 1988; Keller, 1981). The dorsal blastopore lip begins to invaginate and tissues begin to converge towards the midline on the dorsal side. During this time, the dorsal mesoderm moves inward starting from the posterior end of the embryo and moving towards the anterior. The mesoderm during this time makes contact with the overlying ectoderm with the organizer eventually reaching the anterior most dorsal ectoderm during the inward movement (Yanagi et al., 2015). This event is crucial for induction of the nervous system as factors that are expressed in the dorsal mesoderm and endoderm inhibit the formation of an epidermal fate and transform the ectoderm to neural ectoderm.

Bone Morphogenic Protein (BMP) signaling in the non-dorsal ectoderm induces epidermal fate through activation of transcription factors such as *msx1* (Suzuki et al., 1997). The expression of BMP inhibitors in the dorsal mesoderm and endoderm, initiating at the organizer, suppresses BMP signaling in the dorsal ectoderm and prevent the cells from adopting an epidermal fate. These BMP

inhibitors such as *Noggin, chordin,* and *follastatin* are secreted by the dorsal organizer during movements in gastrulation (Brazil et al., 2015; Dudarić et al., 2013). As discussed previously, as the dorsal mesoderm and endoderm move inward the secretion of these BMP antagonists repress epidermal fate and lead to the specification of neuronal phenotypes. This led to the adoption of the "default neural state model" where repression of signaling maintained neural identity while active signaling (BMP) lead to epidermis from the original ectoderm.

In addition to BMP signaling, Wnt signaling is also active in epidermis and is inhibited in neural ectoderm during its specification. Interestingly enough, the leading edge of the blastopore lip, as it is moving to make contact with the anterior-most dorsal ectoderm (fated to give rise to the forebrain) begins to express Wnt inhibitors such as *Cerberus, Frzb* and *dickopft* that inhibit Wnt signaling and stabilizes and anterior fate. The dorsal-posterior, which is still receiving Wnt signaling, is transformed to a more caudal fate of the neural ectoderm from the active Wnt signaling (Constance Lane et al., 2004; Kazanskaya et al., 2000; Kuroda et al., 2004; Leyns et al., 1997; Wang et al., 1997). Thus, it is believed that anterior neural fate is the default ectodermal fate in development and signaling factors transform the neural tissue into posterior fates and transform ectodermal tissue into epidermis.

2.3 Activation Transformation and Opposing Gradients

The two driving hypotheses are that neural tissue activates to become anterior and caudal factors transform the tissue to a more posterior fate while the opposing gradients hypothesis states that factors along the anterior-posterior axis have a specific patterning gradient that induce certain structures (Gamse and Sive, 2000).

Fibroblast Growth Factor (FGF) signaling provides one of the strongest supporting evidences for the activation-transformation hypothesis as FGF overexpression analysis leads to the caudalization of tissue and inhibition of anterior fates. Thus, neural tissue through active FGF signaling is transformed to become posterior (Deimling and Drysdale, 2011; Ota et al., 2009; Pera et al., 2014). Retinoic Acid (RA) is also important in leading to posterior fates as retinoic acid is highest in the posterior and treatment of embryos with retinoic acid leads to the posterization of anterior structures suggesting once again that they have been transformed to posterior fates from active signaling (Paulsen et al., 1994).

The strongest piece of evidence of the activation part of the hypothesis is the expression of *Otx2.* Which is initially, expressed on the leading edge of the dorsal lip and begins to move anteriorly with the invaginations during gastrulation. The early expression of this potent inducer of forebrain development may suggest that it acts as the activator in initially setting up and anterior neural fate (Blitz and Cho, 1995; Gammill and Sive, 1997).

The opposing gradients hypothesis suggests that gradients along the anterior-posterior axis are crucial in establishing a neural fate. *Otx2* serves as another strong piece of evidence in this hypothesis as Cad2, which is expressed in the posterior, opposes its expression along the axis (Chalmers et al., 2000; Houle et al., 2000). This gradient sets up the boundary for where another factor, *Gbx2,* at the midbrain/hindbrain boundary to induce formation of the proper structures (Inoue et al., 2012; Nakayama et al., 2013; Steventon et al., 2012). These opposing gradients leading to the formation of other downstream regulatory genes that properly pattern the axis.

These two hypotheses help explain how the anterior-posterior axis is formed with the anterior being induced early on and active signaling transforming neural cells to a more posterior fate. In addition, opposing gradients from the anterior-most end and the posterior-most end set up patterning that may allow for the stabilization of regional identity and the expression of other factors that are critical for the development of regions that are not in the extremes of the anterior or posterior edge.

2.4 Neural Competency

Despite the presence of these strong inducers and active signals neural tissue must remain competent to receive these signals in order to be able to respond appropriately. This is especially relevant to plasticity as an extended period of competency is necessary in order for cells to respond and change cellular physiology based on the inducing environment. Early studies discovered

that the inducing environment to make for neural identification was expressed over a much larger area than what was eventually fated to become neural. These studies transplanting early gastrula tissue into an early neurula demonstrated that the inducing environment is expressed over a much larger spatial range and longer temporal range than the neural tissue is capable of being able to respond (Albers, 1987). In addition other early experiments revealed that, as development progressed the tissue was less and less able to respond to inducing signals from the underlying mesoderm (Domingo and Keller, 2000). More recently, molecular evidence implicates factors like *Epi1,* expressed on the ventral epidermis prior to gastrulation, and *Xlfc,* expressed along the neural tissue, in determining competence. Epi1, as an example, activates downstream targets that inhibit neural induction (Shapira et al., 2000) while *Xlfc* overexpression increases the area of ectoderm that is competent to become neural (Morgan et al., 1999).

2.5 Summary

Early maternal determinants and sperm entry set up the dorsal ventral axis and initiate the formation of the organizer in the dorsal-posterior of the embryo. Cellular movements initiated by bottle cells of the organizer start gastrulation and cause the invagination of dorsal mesoderm and endoderm to move in first and make contact with the overlaying ectoderm. Inhibition of BMP and Wnt signaling by factors in the dorsal endoderm and mesoderm induce neural identity in addition to Wnt inhibition leading to an anterior neural fate while active Wnt signaling leading to a posterior fate.

The anterior most portion of the neural ectoderm is specified early in gastrulation via activation through *Otx2.* Posterior fates are actively transformed through FGF and RA signaling occurring in high concentrations in more caudal locations. In addition, opposing gradients of gene expression are set up from the anterior and the posterior to provide regional identity to the overlaying ectoderm.

The ectoderm, also, has a role in determining fate through its competency to respond to signals. An important aspect of this is the period and length of competency. While tissues may be specified to adopt a certain fate, they may remain competent to other signals to change their fate as they have yet to become determined or committed to that. This plasticity, while present throughout development, is still poorly understood and the bounds that define competency in cells are still under current investigation. The anterior-posterior axis is specified early as evident by the signaling events occurring extremely early in gastrulation. However, its competency to respond to different signals is present until the onset of neurulation at which point the neural tissue has lost its competency.

3. EXPERIMENTAL OVERVIEW

These findings demonstrate that active signaling induce a posterior neural fate while antagonists and stabilizing factors maintain anterior identity and that the neural tissue must be competent in order to correctly pattern itself in the presence of those signals and factors. The axis is also patterned along through

opposing gradients that act to fine-tune regionalization of the pattern. These findings suggest that neural tissue is specified and that a striking and stable anterior-posterior neural axis is patterned during blastula and gastrula stages. While the determinants and patterning has been extensively studied, the plasticity of this process is very poorly understood. Even though different organisms display different timing and capacities to regulate, it is established that all organisms maintain some plasticity during development. Many transplantation experiments reveal the regulative capacity for tissue to not only compensate for a wound but also compensate for the addition of foreign tissue from a donor. While the regulation may not be fully complete following such perturbations, it is still extremely telling of the presence of plasticity.

As neural plasticity along the anterior-posterior axis is not well established, this project will attempt to determine the regulative capacity of neural tissue following an inversion and transplantation of the neural tissue. This will not only allow for the testing of the plasticity following microsurgery on the neural tissue but will also allow for the testing for the ability of the axis to be able to redifferentiate itself following an inversion.

In order to test for the integration and localization of tissue into the host, a labeled donor will be used with a fluorescent-labeled tag. This will allow for visualization of donor tissue within the unlabeled host embryo. Embryos following transplantation (either inverted axis or control axis) will be fixed and undergo *in situ* hybridization for target genes using digoxigenin-labeled antisense RNA probes in order to analyze spatial and temporal expression of these genes

following surgery. In order to visualize the location of the graft tissue into the host, antibodies specific the fluorescent label will be used to differentiate between host/donor tissue post-*in situ* hybridization.

Embryos will undergo transplants of their presumptive neural tissue at the mid-gastrula and late gastrula stages of development where the anterior-posterior orientation of the graft will match the host (Control) or will be rotated 180°relative to the host (Rotated). Previous studies from the lab have revealed that transplants are able to recover and reach tadpole stages when performed at the mid-gastrula but unable to do so if the transplant is performed at the late-gastrula. Explant analysis using anterior-posterior marker genes along the axis (*XCG-1*, *Otx2, En2, Krox20*) revealed that the expression of the genes is present in the explants derived from mid-gastrula tissue, in addition to terminal differentiation markers of neurotransmitter phenotype (*xGAD*). This reveals that the tissue has already specified to its anterior-posterior fate by mid-gastrula.

Embryos that have had tissue transplanted onto them at either the mid-gastrula stage or the late-gastrula stage will be probed for genes along the anterior-posterior axis (*XCG-1*, *Otx2, En2, Krox20*) to characterize their expression and regulation in embryos that had their tissue transplanted and rotated, had their tissue transplanted but not rotated, or had no surgical manipulation. The gene expression of these marker genes will be compared to test the bounds of regulation following surgery and an inversion of the axis.

Embryos that receive transplants must be able, at the very least, to integrate foreign neural tissue into themselves regardless if the axis is inverted or

not. As the embryo must also integrate a foreign graft, transplantation experiments do not demonstrate the ability of the embryo to heal following a similar surgical removal. These ablations of tissue will elucidate the healing capacity of the neural tissue in the areas surrounding the surgery site.

4. MATERIALS AND METHODS

4.1 Animal Care

All animal use protocols were approved by the Institutional Animal Care and Use Committee (IACUC).

Adult *Xenopus* were maintained in tanks with a maximum of 12 males or 6 females per tank. All *Xenopus* were kept in well-water with NaCl added to achieve an approximate concentration of 20 mM. Water temperature was kept between 18°C-22°C. Females and males were housed in separate tanks and water was changed after each feeding by flushing over a 4-6 hour time span. Adult frogs were fed three times weekly and allowed at least an hour to eat before any flushing began.

All embryos were obtained from natural matings carried out in a transparent mating bucket suspended with a mesh bottom through which fertilized embryos and oocytes would fall. The mating bucket was suspended, in water, above the bottom of a tank with a plastic tray underneath it to collect the embryos and oocytes. Matings were induced with injection of human chorionic gonadotropin (HCG) solution into the dorsal lymph sac using a 27½ gauge needle and a 1 ml syringe approximately 10-12 hours prior to desired embryo

collection. Females were injected with 0.7 ml (700 U) and males with 0.5 ml (500 U) (Sive et al., 2007).

Embryos and oocytes, which fell through onto the plastic tray, were collected by pulling out the tray containing them. All solution was decanted and replaced with 100 ml of a 2% L-cysteine solution in 0.1X Marc's Modified Ringers (MMR) with a pH adjusted to 7.8-8.0 with NaOH. The tray was gently rocked until the jelly coats had been completely removed (No longer than 5 minutes). Embryos were washed three times in 0.1X MMR with a concentration of 50 µg/mL gentamicin and were placed in 100 mm glass plates in 0.1X MMR with gentamicin in which they were maintained at a density of approximately 50-70 embryos. Approximately 2 hours after collection, embryos that appeared unfertilized or those that were not dividing properly were discarded by placing them in a solution of 10-15% ethanol before properly disposing of them. If embryos were older than neurula stages they were first anaesthetized in a solution of MS222 (Sigma) and then placed in the ethanol. Solution was replaced with fresh solution daily and necrotic embryos were removed each time. *Xenopus laevis* embryos were kept at 14°C-22°C during their development (Sive et al., 2007a). All embryos were staged according to stages described by Nieuwkoop and Faber (Nieuwkoop and Faber, 1994).

4.2 *Embryo Microinjection*

Needles for microinjection were pulled from 7" Drummond capillaries (using a Narishige model PB-7 vertical needle puller). Capillary tubes were pulled to 5-10 µm using the heating coil. The resulting needles were broken to a

desired diameter (15 μm-30 μm) using a single ply of toilet paper stretched tightly over a beaker, where the needle was pushed through to break the tip, or using Dumont No.5 fine forceps to break the tip off the needle. Once broken the needle tip was checked using an objective micrometer to ensure that it was of the right diameter to inject, that it was properly broken, and would not be clogged.

Stock solutions of the lysine-fixable fluorescein-linked dextran or rhodamine-linked dextran were made as 10% and 4% solutions(Sive et al., 2000), respectively by diluting in nuclease-free water. Afterwards, the solutions were stored in light-protective tubes at -20°C until time for injection.

Embryos were injected using the Drummond Nanoject 2.0. At the 2-cell stage*, Xenopus laevis* blastomeres were injected with 4.6 nl containing 4% rhodamine-linked dextran or 10% fluorescein linked dextran into each blastomere (Sive et al., 2000). 2-cell staged embryos were transferred to dish with a clay bottom with wells or to hold embryos in place containing a 4% ficoll (GE Healthcare) solution in 1/3X MMR. Each embryo was injected into one and then the other blastomere. Embryos that reached the 4-cell stage or beyond prior to being injected were ignored and not included in the future plate of injected embryos. Embryos post-injection were kept in a plate containing 4% ficoll in 1/3X MMR. 2 hours after being in the 1/3X MMR, embryos were transferred to plates containing 0.1X MMR with 4% ficoll. 6 hours post the initial solution change to 0.1X MMR with 4% ficoll, embryos were sorted to remove any necrotic or improperly dividing embryos and then embryos that remained were transferred to fresh solution of 0.1X MMR with 4% ficoll. 12 hours after the second solution

change and before the embryos reached the mid-gastrula stage of development (Stage 11.5), necrotic and improperly dividing embryos were once again removed and remaining embryos were screened using a UV burner equipped stereoscope to ensure proper injection by observing rhodamine or fluorescein fluorescence. Embryos were discarded if there was no presence of fluorescence or the fluorescence signal did not appear throughout the entire embryo such as in cases of accidental unilateral injections into the blastomeres.

4.3 Transplantation Experiments

Using the rhodamine or fluorescein injected embryos from above as donors and uninjected and unlabeled embryos as hosts. The vitelline membrane from the embryos was removed prior to the surgery using Dumont No.5 fine forceps without damaging the underlying tissue of the embryo. The embryos were transferred in a clay-bottomed dish in 1/3XMMR with 4% ficoll. The embryos were placed snuggly into clay wells, indented into the clay using dull forceps. The embryos were placed with the presumptive neural ectoderm facing up and the blastopores pointing downward so the embryos are in the same anterior-posterior orientation. Dissection needles to aid with the surgery were made using the same settings on the Narishige model PB-7 vertical needle puller, but used thicker and larger diameter capillary tubes to provide a more stable dissection tool. The tip of the needles used for dissection was not broken and maintained for multiple transplantations. The medial 50% along the anterior-posterior axis of presumptive neural ectoderm during gastrulation was removed first by making incisions along the lateral edges using dissection needles, being

aware not to cut into the underlying mesodermal layer, then by using fine forceps and the dissection needles to cut along the edges approximately ¼ mm above the blastopore and approximately ¼ mm below the anterior most edge of the dorsal ectoderm. The explant was lifted up using the sides of the dissection needle and was inspected to ensure there was no mesodermal contamination. The host embryo had its neural ectoderm removed and replaced with the neural ectoderm of the fluorescently labeled donor embryo. During this transplantation, the explant was placed onto the host that had its tissue previously removed as discussed, so that the explant's original anterior-posterior axis matched the host's anterior-posterior axis or was rotated 180° relative to the host's axis. A glass chip was placed over the transplant to facilitate incorporation. When the axis orientation matched the host's, the embryos were labeled as "Control Transplants." When the axis orientation was rotated relative to the host, the embryos were labeled as "Rotated Transplants." 2-3 hours after transplantation, the glass chip was removed and the embryos were transferred to 0.1X MMR with 4% ficoll and then 6 hours afterwards were replaced into fresh 0.1X MMR with 4% ficoll. Embryos were allowed to grow up to stage 30 before being fixed in a formaldehyde-based fixative for subsequent analysis.

4.4 Ablation Experiments

Ablation experiments were performed by removing the presumptive neural tissue and exposing the underlying mesoderm without the addition of any explant or tissue as replacement. Immediately after removal of the presumptive neural tissue, embryos with part of their neural tissue ablated were imaged for 30

minutes under a stereoscope during which an image was captured every 30 seconds. Embryos undergoing ablations underwent the same solution changes as transplanted embryos and were allowed to grow up to stage 30 before being fixed.

4.5 Embryo Fixation

In order to fix *Xenopus* embryos for *in situ* hybridization experiments, embryos older than stage 20 were first anesthetized in a MS222 solution (Sigma) and then quickly rinsed in a 0.1X MMR. Embryos were then transferred to 5 ml glass vials with as little solution as possible. The vial was then filled with 1X MEMFA and rocked horizontally at room temperature for 90 minutes or at 4°C for 10-13 hours (Sive et al., 2000a). The embryos were then dehydrated with two washes in 100% ethanol each lasting after which the embryos were stored at -20°C in ethanol.

4.6 Cloning of Krox20

The probe used for *Krox20* for *in situ* hybridization experiments was synthesized from cloned cDNA fragments of the gene of interest.
Primers used for cloning were designed from known RNA *Xenopus laevis* sequences using Primer3Plus with an optimum T_m of 60°C and an optimum GC% of 50%. Acceptable primers anneal with 20-23 bp, have a T_m of 56°C-61°C, a GC% of 40%-60%, and pass the default self-complementarity parameters of Primer3Plus. Primers used to clone of *Krox20* are: Forward: 5'-AACCGCCCCAGTAAGAAC-3' Reverse: 5'– GTGTCAGCCTGTCCTGTTAG-3'.

Primer oligonucleotides arrive as lyophilized pellets which were resuspended in 1X TE to create a 1mM stock by adding 1 µl 1X TE for each nanomole of oligonucleotide. Subsequent 10^{-1} and 10^{-2} serial dilutions were made from this stock in nuclease free water.

cDNA used for cloning was synthesized from total RNA extracted from st. 18 and 30 *Xenopus laevis* embryos using Tri-reagent. cDNA was synthesized in 20 µl reactions using the iScript kit (BioRad) according to manufacturer instructions with 1 µg of total RNA. 1 µl of the resulting cDNA (~50 ng) was used for each PCR reaction. PCR reactions were carried out using Platinum Taq High Fidelity. Platinum Taq High Fidelity contains both DNA taq polymerase and proofreading enzymes. All reactions were carried out according to manufacturer protocols for each enzyme using 35 cycles. An annealing temperature of 60°C was used routinely for these targets. The extension phase of each cycle was extended to allow 1 minute per kilobase of the desired region of interest to be cloned. The presence of correctly sized amplicons was verified using agarose gel electrophoresis.

Amplified gene fragments were ligated into the Strataclone pSC-A amp/kan plasmid vector using the Strataclone PCR cloning kit (Stratagene) following manufacturer instructions. These ligations were then transformed into Strataclone SoloPack Competent cells according to manufacturer instructions and the transformants were plated on Luria Broth (LB)-agar plates containing 50 µg/ml ampicillin with 40 µl 2% x-gal for blue-white colony screening. After 12-16 hours of incubation at 37°C, white colonies were used to inoculate miniprep

cultures each containing about 3-5mL of LB which were grown in a shaking 37°C incubator at 225-250 RPM for 10-14 hours. Bacterial glycerol stocks were created by mixing 500 µl sterile glycerol with 500 µl turbid culture and were stored at -20*C. Plasmid DNA was isolated using the Promega Wizard Plus Miniprep DNA Purification System according to manufacturer instructions. The insert was sequenced using an ABI 3500 Genetic analyzer using the M13 forward and reverse universal sequencing primers.

4.7 Restriction Digest

Restriction digests were typically composed of 0.5 µg of DNA, 1X of the appropriate restriction enzyme buffer based on the manufacturer (Promega), 5 units of the appropriate restriction enzyme (Promega) and sdd water to bring the total volume to 20 µl. Digests were incubated for 1-2 hours at the appropriate temperature and 4 µl of 6X DNA dye was added to the mixture. Samples were run on a 1% mini agarose gel composed of 50 ml 1X Tris Acetate EDTA (TAE), and 0.5 g agarose, which was heated in a microwave until all agarose was dissolved. To this mixture, 3.5 µl of 10 mg/ml stock ethidium bromide was added for a final concentration of 0.5 µg/ml. Gels were covered in 1X TAE and run for 30 minutes at 160-170 volts with a maximum current of 500 milliamps. The first lane of each gel was loaded with 10 µl of 1 kb+ ladder (Invitrogen) in 1X DNA dye (Green and Sambrook, 2012). The remaining lanes were loaded with 12 µl of the digest (including the dye). Adjacent to digested samples, undigested 12 µl samples consisting of 0.5 µg DNA, 2 µl 6X dye, and sdd water (to 12 µl) were run as a control for comparison. Gels were visualized using ultraviolet

transillumination in a FluorChem light box (Alpha Innotech) and photographed and annotated using FluorChem HD2 software.

4.8 Plamid Purification of All Constructs

Other constructs used (See Table 1) were already present as glycerol stocks and have been received as stocks by other labs or had been cloned by previous lab members.

10 µl of glycerol stock containing the transformed bacteria with the clone of interest was added into an Erlenmeyer flask containing 150 mL of LB. The flask was placed in a 37°C shaker shaking at 200-250 RPM for approximately 12 hours. The culture was placed in a centrifuge tube and spun down at 5,500 RPM on the F14 Fiberlite rotor (Thermo Scientific) to pellet the bacterial cells. The supernatant was removed and the bacterial cells were subsequent lysed, and underwent washes and column filtration using the Machery-Nagel Midiprep Plasmid Purification kit following the manufacturer's protocol. The DNA, once purified and pelleted, was finally eluted in nuclease-free water (Promega). DNA was quantified using spectrophotometry (Nanodrop) and 0.5 µg of DNA was used in a restriction digest using enzymes that cut out the insert (described previously) to check for the presence of the target clone in the plasmid.

4.9 Linearization

To linearize plasmid DNA for subsequent use in transcription as a template, digests were set up containing 100 µL per reaction. Each eppendorf tube contained: 1X restriction enzyme buffer 20-30 µg of plasmid DNA, 20-30 units appropriate restriction enzyme (Depending on enzymatic activity) nuclease

free water was added to have 100 µL total per reaction. Digests were incubated at the appropriate enzymatic temperature for 2-4 hours. DNA was extracted by adding an equal volume of phenol/chloroform: isoamyl alcohol (25:24:1) (PC:IAA) then mixed and centrifuged in a microcentrifuge for 5 minutes at 14,000 RPM. The organic layer was removed and discarded. The remaining aqueous layer was extracted with an equal volume of chloroform: isoamyl alcohol (24:1) (C:IAA) and then mixed and centrifuged down in a microcentrifuge tube. The aqueous layer was removed and placed into a new eppendorf and the remaining eppendorf with the organic layer was discarded. 1/10 volume of 3M sodium acetate was subsequently added to precipitate linearized DNA followed by 2 volumes of cold 100% ethanol (ethanol was stored at -20°C before being added). Linearized solution was frozen at -80°C for at least 15 minutes. Linearized reaction was centrifuged in a refrigerated centrifuge (Eppendorf) at 4°C for 20 minutes at 14,000RPM. Supernatant was removed and DNA pellet was washed with 200 µL of fresh 70% ethanol and centrifuged in the refrigerated centrifuge at 4°C for 5 minutes at 14,000RPM. Supernatant was discarded and the DNA pellet was air dried by leaving the cap open for 5-7 minutes and then eluted in 20 µL 1X Tris EDTA (TE) 10mM and 1mM respectively. Linearized DNA was run out on a 1% agarose gel (as described in section 4.7) to ensure that it was linearized properly.

4.10 Probe Synthesis

Labeled antisense RNA probes were synthesized *in vitro* using the bacteriophage RNA polymerase T3 or T7 from linearized plasmid DNA. 50 µl

transcription reactions included 4µg of linearized template DNA (2-8 µl depending on plasmid concetration), 1X transcription buffer (Promega), 10mM Dithiothreitol (DTT), 750 µM each of rGTP, rCTP, and rATP, 488 µM rUTP, 262 µM digoxigenin-11 rUTP (Roche), 10 units RNAsin (Promega), and 25 units of the appropriate RNA polymerase (Promega). Transcription reactions were incubated 1 hour at 37°C, an additional 25 units of RNA polymerase was added and reactions were incubated an additional hour. 1 µl RQ1 DNAse (Promega) was added to degrade the linear DNA template that was used for the reaction and the reaction was incubated at 37°C for 10 minutes to facilitate this.

Immediately following template degradation, 30 µl LiCl precipitation solution for a final concentration of approximately 2.5M LiCL (Stock: Ambion – 7.5M LiCl, 1mM EDTA) was added and the reaction was mixed well by pipetting and stored at -20°C for at least1 hour to overnight to precipitate the RNA. The reactions were then centrifuged 25 minutes at 14,000RPM using the refrigerated centrifuge (Eppendorf) at 4°C to pellet RNA probe. The supernatant was discarded and the pellet washed with 200 µl 70% ethanol. The reactions were centrifuged 5 minutes at 14,000RPM at 4°C, the supernatant was discarded, and the pellet was air dried, similar to the linearization (See section 4.9) for 3-5 minutes. The pellet was then brought up in 20 µl nuclease free water (Promega) and analyzed by agarose gel electrophoresis and spectrophotometry (Nanodrop). Probes used produced a clean band or, occasionally due to RNA secondary structures, a doublet on an agarose gel. Probes were brought up to a stock concentration of 10X (10 ng/µl) with ISH buffer (Sive et al., 2000b) which

immediately stabilizes the RNA. Stock probes were stored at -20°C and were diluted to 1X concentration (1 ng/µl) in ISH buffer prior to use.

Table 1. Clones utilized for synthesis of ISH antisense RNA probes.

Gene	Reference	Vector	Resistance	Insert Site	Insert Length	Linearization	Transcription
XCG-1	Sive et al. 1998	pCS2	AMP	EcoR1	650 bp	Not1 for sense transcript	T3 for sense transcript
Otx2	Blitz et al. 1995	pBluescript SK-	AMP	Not1, EcoR1	2.2 kb	Not1 for antisense transcript	T7 for antisense transcript
En2	Ali Hemmati-Brivanlou 1991	pBluescript KS+	AMP	EcoR1	1.5kp	Xba1 for antisense transcript	T3 for antisense transcript
Krox20	Nieto et al. 1991	pSC-A	AMP/KAN	EcoR1	445 bp	BamH1 for antisense transcript	T3 for antisense transcript

4.11 Double Chromogenic Whole-mount in situ hybridization

Whole-mount *in situ* hybridization was performed as described by Sive et al. Embryos were rehydrated by successive 5-minute washes in 100% ethanol, 75% ethanol/25% sdd water, 50% ethanol/50% sdd water, 25% ethanol/75% PTw, and 100% PTw. Following rehydration embryos were washed three additional times for 5 minutes in PTw. To permeabilize embryos for hybridization, embryos were treated in 1 ml 10 µg/ml Proteinase K (Promega) in sdd water for 30 minutes at room temperature with nutation. A longer Proteinase K treatment was used to increase sensitivity in comparison to the original protocol, which called for a 15 minute incubation.

Following rinses in triethanolamine and treatment with acetic anhydride, embryos were rinsed twice for 5 minutes in PTw before re-fixing in 4% paraformaldehyde. Residual paraformaldehyde was rinsed away using three 5-minute washes in PTw. Embryos were prehybridized at 60°C for a minimum of 6 hours in ISH buffer. Embryos were then incubated with 500 µl of diluted probe (1X concentration 1ng/µL) as described above for 12-15 hours. Probes were used in a total of no more than three *in situ* hybridization experiments and then a new diluted probe had to be used. Following removal of the probe, embryos were incubated in 500 µl buffer for 10 minutes at 60°C.

Following RNAse treatment to remove unbound probe, embryos were washed twice in 2X SSC for 5 minutes at room temperature followed by two 30-minute washes in 0.2X SSC at 60°C. Embryos were then washed twice in maleic acid buffer (MAB) for 15 minutes. MAB was removed and embryos were incubated in 500 µl 2% BMB blocking reagent (Roche) in MAB for 1 hour at room temperature with nutation and then moved to 4°C before addition of the antibody. Embryos were then incubated in 500 µl 2% BMB in MAB containing a 1:2000 dilution of anti-digoxigenin antibody (against the dig-labeled probe) coupled to alkaline phosphatase at 4°C for 12-15 hours.

Excess antibody was removed with five washes in MAB each lasting for at least 1 hour. The last wash was typically done at 4°C overnight to reduce background staining. Following the MAB washes, embryos were washed twice for 5 minutes in alkaline phosphatase buffer (AP Buffer) (Sive et al., 2000b). All solution was removed and replaced with 1 ml AP buffer containing the color

substrates: 4.5 µl nitro-blue tetrazolium (NBT) and 3.5 µl 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Promega). Vials were incubated at room temperature until sufficient signal developed and completed color reactions were stopped by removal of the AP buffer containing the color substrate and the addition of MAB containing 10mM EDTA. The embryos were let to incubate at 65 ^{O}C to stop the color reaction. Embryos were then dehydrated by successive 5-minute washes in 25%Methanol/75%MAB, 50%Methanol/50%MAB, 75%Methanol/25%MAB, and 100% methanol. Promptly after, embryos were rehydrated by 5-minute successive washes 75%Methanol/25%MAB, 50%Methanol/50%MAB, 25%Methanol/75%MAB, and 100% MAB. Embryos were then washed twice in MAB for 15 minutes each and then blocked for 1 hour at room temperature in 2% Blocking reagent in MAB and then moved to 4°C before addition of the antibody. Embryos were then incubated in 500 µl 2% BMB in MAB containing a 1:2000 dilution of anti-fluorescein antibody (against the injected fluorescein-linked dextran present in the graft from the donor embryo) coupled to alkaline phosphatase at 4°C for 12-15 hours. Excess antibody was removed with five washes in MAB each lasting for at least 1 hour. Following the MAB washes, embryos were washed twice for 5 minutes in alkaline phosphatase buffer. All solution was removed and replaced with 1 ml AP buffer containing 3.5 µl of just BCIP. Vials were incubated at room temperature until sufficient signal developed at which point the AP buffer was removed and replaced with 1X MEMFA and left nutating overnight at 4 ^{O}C. Following refixing, embryos were rinsed once in 1X

PBS at room temperature for 5 minutes. Embryos were then stored at 4°C in fresh 1X PBS.

4.12 Whole-mount images

Embryos were photographed for whole mount photography using an Olympus SZH10 microscope and an Olympus DP71 camera controlled by DP controller and DP manager software (Olympus). Bright field photographs were taken against a white background at magnitudes of 25 to 30X while fluorescent photographs were taken against a black background of the same magnitudes. Whole images were adjusted for color, brightness, and contrast using Adobe Photoshop CS3.

5. RESULTS

5.1 Embryos are able to recover mid-gastrula axis inversion and transplants

In order to test the regulative capacity of embryos being able to recover post-transplantation and axis inversion, presumptive neural ectoderm was removed from fluorescently labeled donor embryos and transplanted onto unlabeled hosts. The embryos then were allowed to develop until sibling embryos that were not surgically manipulated reached stage 30 and then the embryos were imaged to investigate incorporation of the graft and phenotypic regulative capacity. Regardless of whether the axis was inverted or not, the embryos were able to recover and appear to be morphologically normal. Not only were they similar to each other in control and rotated but they also were similar to un-manipulated sibling embryos (Figure 1). Out of 126 embryos that underwent

rotated transplants, 110 survived and had normal morphology as compared to siblings (87.3%) in addition to 46 out of 47 embryos (97.9%) that had undergone control transplants had normal morphology (Table 2). Indicating that embryos had the ability to recover post surgery.

Table 2. Survivability and Morphology of Embryos at Mid-Gastrula Transplants

Stage 11.5 to 11.5 Transplants	Number of Embryos with Normal Morphology	Number of Embryos with Abnormal Morphology	Number of Embryos that did not survive
Control Transplant	46/47 (97.9%)	1/47 (2.1%)	0/47 (0%)
Rotated Transplant	110/126 (87.3%)	15/126 (11.9%)	1/126 (0.8%)

5.2 Embryos are not able to recover late-gastrula axis inversion but are still able to recover from transplantation.

In order to test the temporal bounds of regulation, transplants were performed at late-gastrula stages (12.5) and the axis was inverted or maintained orientation relative to the host. The embryos then were allowed to develop until sibling embryos that were not surgically manipulated reached stage 30 and then the embryos were imaged to investigate incorporation of the graft and phenotypic regulative capacity. Embryos that had a rotated axis were unable to recover from the rotation and would often not survive into swimming tadpole stages or have abnormal morphology. Embryos whose anterior-posterior axis was rotated at stage 12.5 would often show delays in neural tube closure in addition to drastic bends and curls at the site of the incorporation of the graft and, in some cases,

the lack of anterior morphology such as a brain or eye (Figure 2). Interestingly, embryos would not show swimming response when gently poked unlike the sibling embryos.

However, embryos in which tissue was still transplanted but not rotated developed similarly to siblings and morphologically normally. They were much more likely to survive into later stages in comparison to the late gastrula embryos in which the axis was rotated (Figure 3).

37 out of 55 (67.3%) of embryos that had their axis rotated did not survive into swimming tadpole stages with the vast majority that did survive showing abnormal morphology. 16 out of 26 (61.5%) embryos that received a non-rotated transplant had normal morphology as compared to non-surgically manipulated siblings (Table 3).

Table 3. Survivability and Morphology of Embryos at Late-Gastrula Transplants

Stage 12.5 to 12.5 Transplants	Number of Embryos with Normal Morphology	Number of Embryos with Abnormal Morphology	Number of Embryos that did not survive
Control Transplant	16/26 (61.5%)	10/26 (38.5%)	0/47 (0%)
Rotated Transplant	1/55 (1.8%)	17/55 (30.9%)	37/55 (67.3%)

5.3 Embryos at late and mid-gastrula are able to recover from ablations of neural ectoderm.

In order to investigate general healing capacity as a factor as opposed to incorporation of a graft and the regulation of that graft into the host inducing environment, ablations of the neural ectoderm were utilized to visualize the capacity to heal from a would. Regardless of whether the ablation was performed at mid or late gastrula stages, the embryo would be able to recover. Contractile-like would appear, bringing the edges of the wound closer together until the initially exposed mesoderm would be covered up by ectoderm. The embryos would develop normal morphology as compared to non-manipulated sibling embryos (Figure 4). Interestingly, if the underlying mesoderm along with the neural ectoderm was removed, exposing the endoderm, the embryo would not be able to recover and endoderm would push outward and eventually (Typically within an hour) the embryo would become necrotic and cease development (Data not shown).

Table 4. Survivability and Morphology Ablations

Ablations	Number of Embryos with Normal Morphology	Number of Embryos with Abnormal Morphology	Number of Embryos that did not survive
Mid-Gastrula Ablations	16/17 (94.1%)	0/17 (0%)	1/17 (5.9%)
Late-Gastrula Ablations	7/9 (77.8%)	1/9 (11.1%)	1/9 (11.1%)

5.4 Marker Genes along the anterior-posterior axis are able regulate in mid-gastrula rotations and transplants

Firstly, using marker genes along the anterior-posterior axis that was specified during gastrulation required the knowledge of the endogenous expression of these markers in non-manipulated sibling embryos. Figure 5 offers images to visualize endogenous expression through the purple color precipitate formed where the genes are expressed. *XCG-1* is expressed in the cement gland and delineates a part of dorsal ectoderm that is not neural. *Otx2* is expressed in the forebrain, midbrain, and the eye and is critical for the formation of an anterior fate during development. *En2* is expressed at the midbrain/hindbrain boundary along the central nervous system, and finally *Krox20* is expressed in two discrete bands of rhombomeres 3 and 5 in the hindbrain.

Embryos that had undergone transplant operations first underwent an NBT/BCIP color reaction catalyzed by alkaline phosphatase bound to an antibody on targeting digoxigenin labeled antisense probe. Then, the reaction was stopped and the protein and antibody denatured before addition of an anti-fluorescein antibody, linked to alkaline phosphatase, to bind to fluorescein present in the graft. A second color reaction was catalyzed using only BCIP leading to the production of a turquoise color precipitate.

` *XCG-1* was present in the cement gland of rotated and not-rotated transplanted tissue. Its expression in surgically manipulated embryos mimicked the endogenous expression of siblings and was never present in ectopic areas in transplanted tissue at the mid-gastrula transplanted embryos (Figure 6). All 6

transplanted and rotated embryos that underwent *in situ* hybridization had proper expression of *XCG-1* as compared to sibling and control transplanted embryos (Table 5).

Otx2 was present in forebrain and the eye in transplanted embryos. Control transplanted embryos always mimicked the expression of sibling embryos (Figure 7). However, in a few cases, of the transplanted embryos (2/8) the expression of *Otx2* would not appear to be strongly expressed in the eye or would extend more posteriorly in comparison to siblings and control transplants (Table 5).

En2 was not as strong of a probe in comparison to the others and thus embryos developed more background staining from the color reaction. Nevertheless, signal could still be differentiated from background and *En2* would be present in the midbrain/hindbrain boundary in rotated transplants (Figure 8). In one instance the staining of *En2* was undetectable in a rotated embryo (Table 5).

Krox20 was present in the rhombomeres of the siblings and controls in all instances. Similarly, rotated transplanted embryos had proper expression of *Krox20* in the hindbrain (Figure 9). However, in one case, *Krox20* was present more anteriorly towards the forebrain in a very small amount (ie there was little staining in that area but still detectable) (Table 5).

Table 5. Gene Expression of Mid-Gastrula Rotated Transplants

Genes Mid-Gastrula Transplants	Number of Embryos that Express the Gene Similar to Siblings/Controls	Number of Embryos that expressed the genes in ectopic areas	Number of Embryos that did not express the gene
XCG-1	6/6 (100%)	0/6 (0%)	0/6 (0%)
Otx2	6/8 (75%)	2/8 (25%)	0/8 (0%)
En2	6/7 (85.7%)	0/7 (0%)	1/7 (14.3%)
Krox20	7/8 (87.5%)	1/8 (12.5%)	0/8 (0%)

5.5 Neural Marker Genes are unable to regulate at late gastrula transplants

In hopes of investigating the gene expression patterns that lead to a lack of regulation in the late-gastrula transplants, stage 12.5 – 12.5 transplants were assayed for marker gene expression using whole mount *in situ* hybridization. In the majority of cases, the control transplanted embryos had similar expression patterns of the investigated marker genes as compared to non-manipulated siblings. However, none of the rotated transplants at the late gastrula stage showed proper expression of the neural marker genes (*Otx2, En2,* and *Krox20*) (Table 6). While *XCG-1,* seemed to be expressed normally, it never co-localized with the graft indicating that that it was not the grafted tissue that was induced to express *XCG-1.* Despite allowing the color reaction, to go on for longer than control and sibling embryos in the same *in situ* hybridization experiment, the late-

gastrula rotated transplants were unable to regulate the expression of the neural marker genes and (Figure 10).

Table 6. Gene Expression of Late-Gastrula Rotated Transplants

Genes Late-Gastrula Transplants	Number of Embryos that Express the Gene Similar to Siblings/Controls	Number of Embryos that expressed the genes in ectopic areas	Number of Embryos that did not express the gene
XCG-1	3/4 (75%)	0/4 (0%)	1/4 (25%)
Otx2	0/3 (0%)	0/3 (0%)	3/3 (100%)
En2	0/4 (0%)	1/4 (25%)	3/4 (75%)
Krox20	0/6 (0%)	2/6 (33.3%)	4/6 (66.7%)

Figure 1. Mid-Gastrula Transplants

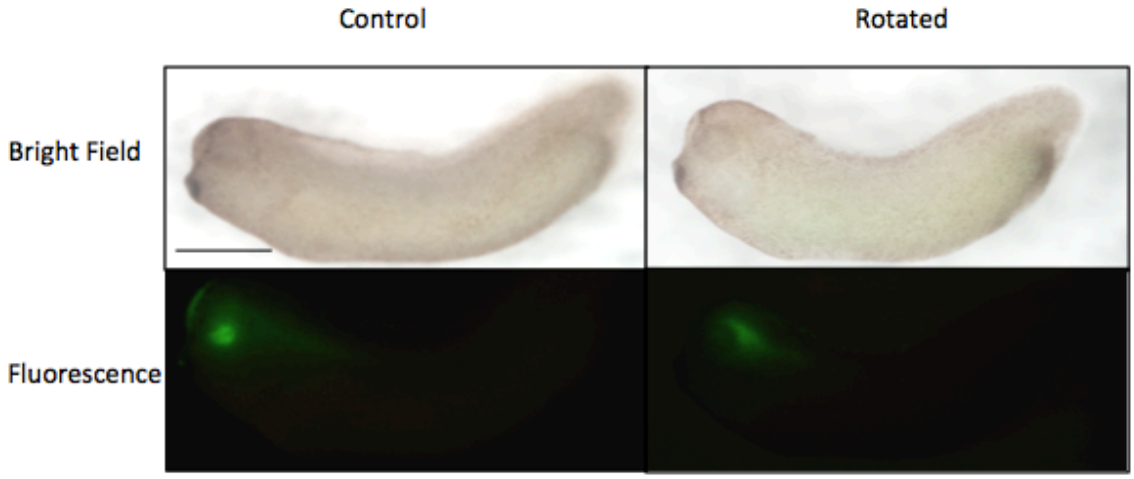

Figure 1. Donor embryos were injected with fluorescein-linked dextran and had their presumptive neural tissue removed and transplanted onto an unlabeled host at stage 11.5 (Mid-Gastrula). The tissue was either rotated relative to the host's anterior-posterior axis (Rotated) or maintained its orientation (Control). Embryos were fixed in swimming tadpole stages and imaged under bright field to visualize and assess the entire embryo of its morphology and under a UV burner through a filter to visualize incorporation of the graft via the appearance of fluorescence indicating presence of fluorescein from the donor tissue. Scale bars are approximately 1mm.

Figure 2. Late-Gastrula Rotated Transplant

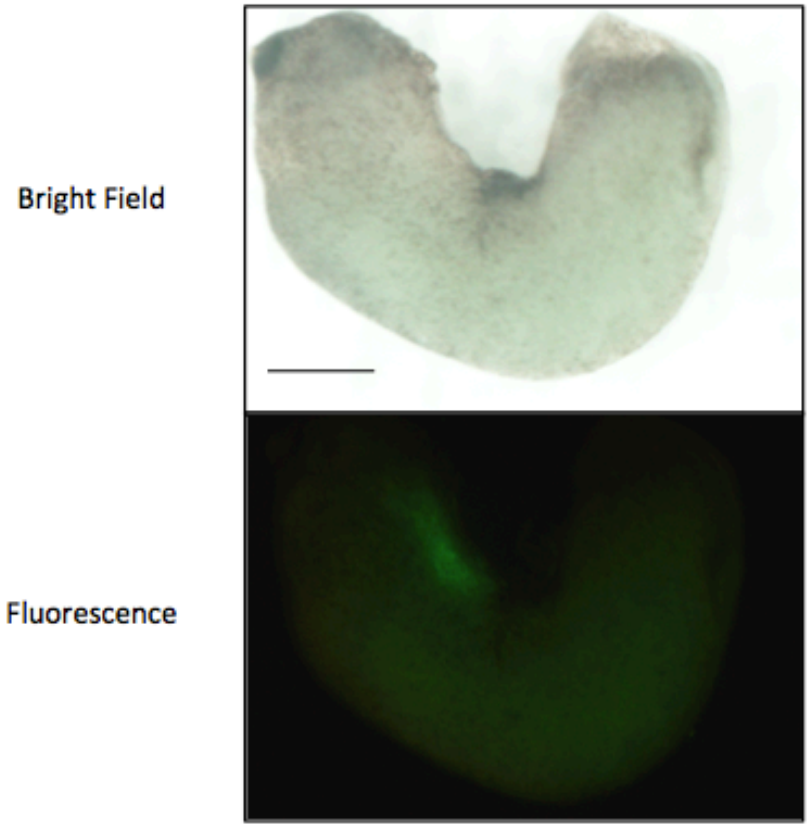

Figure 2. Donor embryos were injected with fluorescein-linked dextran and had their presumptive neural tissue removed and transplanted onto an unlabeled host at stage 12.5 (Mid-Gastrula). The tissue was rotated relative to the host's anterior-posterior axis. Embryos were fixed when un-manipulated siblings reached swimming tadpole stages and imaged under bright field to visualize and assess the entire embryo of its morphology and under a UV burner through a filter to visualize incorporation of the graft via the appearance of fluorescence indicating presence of fluorescein from the donor tissue. Scale bars are approximately 1mm.

Figure 3. Late-Gastrula Control Transplant

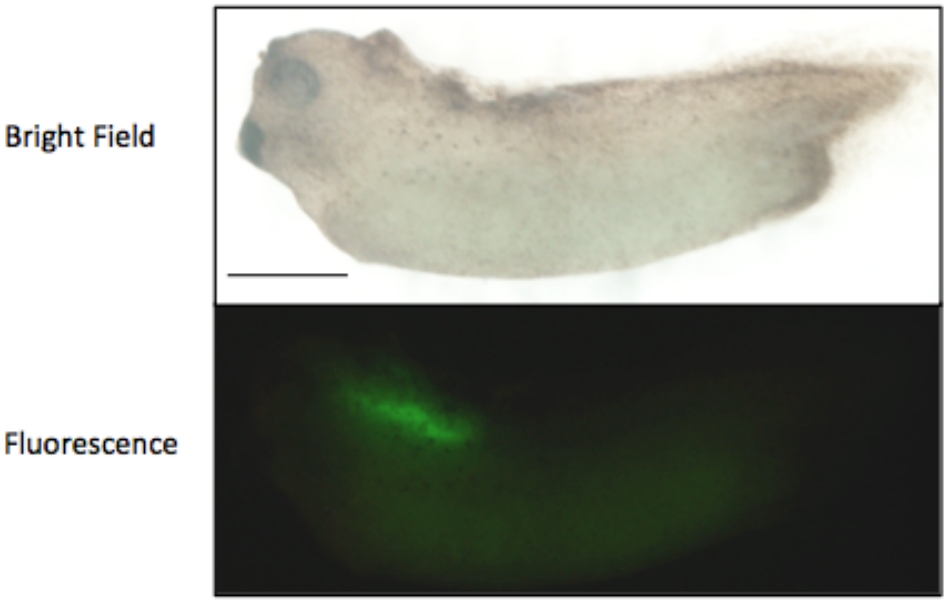

Figure 3. Donor embryos were injected with fluorescein-linked dextran and had their presumptive neural tissue removed and transplanted onto an unlabeled host at stage 12.5 (Mid-Gastrula). The tissue maintained orientation relative to the host's anterior posterior axis. Embryos were fixed when un-manipulated siblings reached swimming tadpole stages and imaged under bright field to visualize and assess the entire embryo of its morphology and under a UV burner through a filter to visualize incorporation of the graft via the appearance of fluorescence indicating presence of fluorescein from the donor tissue. Scale bars are approximately 1mm.

Figure 4. Ablation Recovery

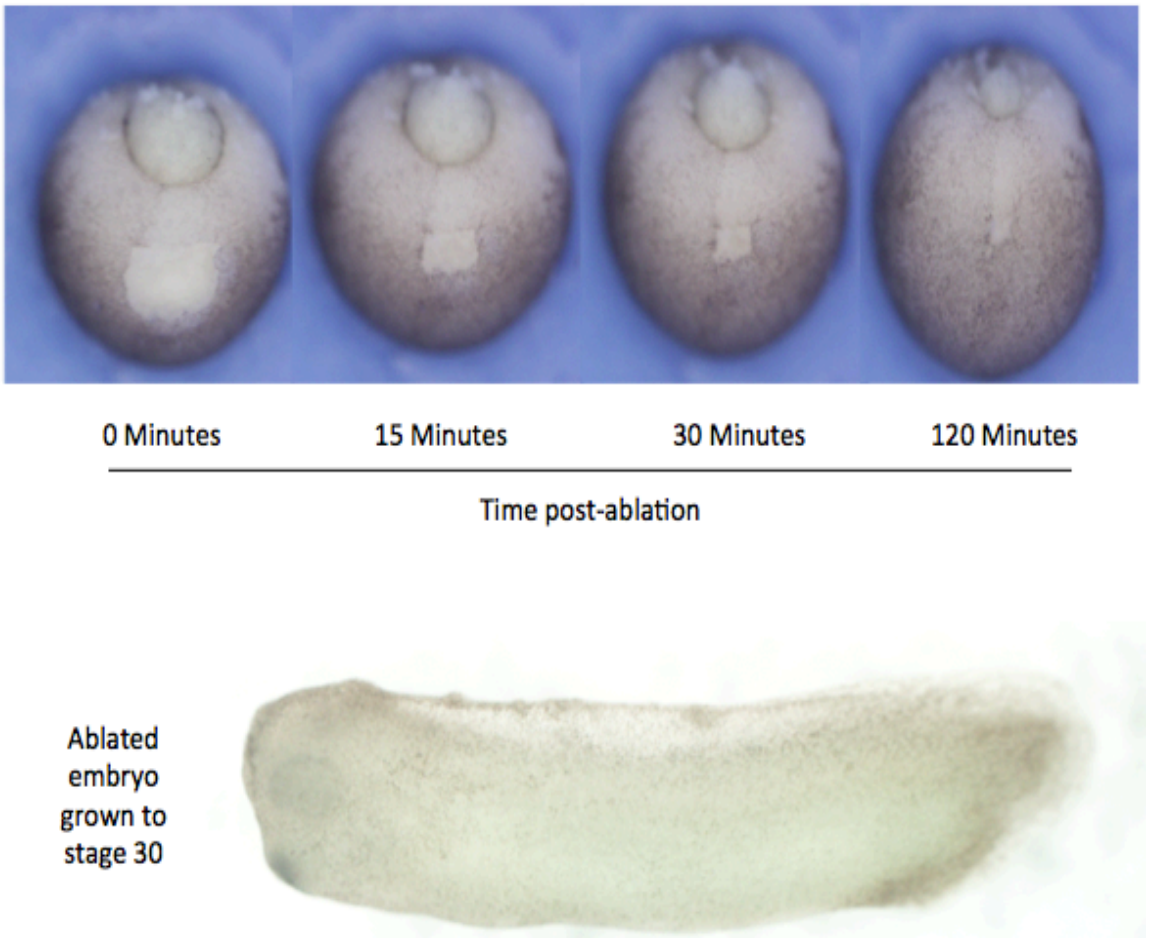

Figure 4. Presumptive neural tissue was removed, but not replaced in embryos at the mid-gastrula and late gastrula stage. Time-lapse images were taken every 30 seconds for 30 minute time-window immediately after ablation of the presumptive neural tissue. Another image was taken 2 hour post-ablation to visualize recovery. The embryo was finally grown to swimming tadpole stages at stage 30 and fixed and imaged to assess for any morphological changes.

Figure 5. Endogenous Expression of Marker Genes

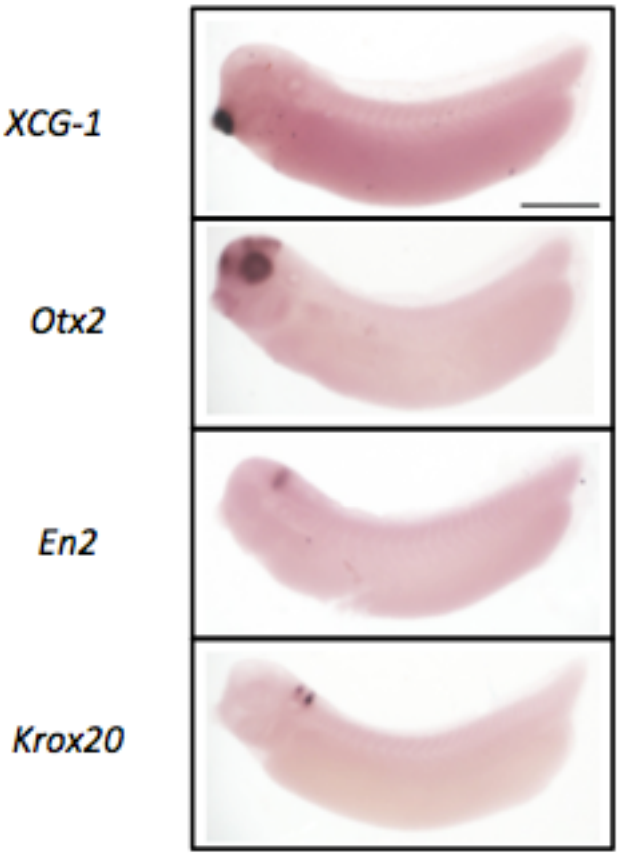

Figure 5. Embryos were fixed at swimming tadpole stage and assayed for *XCG-1, Otx2, En2,* and *Krox20* expression using whole mount *in situ* hybridization to get a view of endogenous expression. Precipitation of a purple color substrate is indicative of expression of the gene. Scale bar represents approximately 1 mm.

Figure 6. Expression of *XCG-1* in Mid-Gastrula Transplants

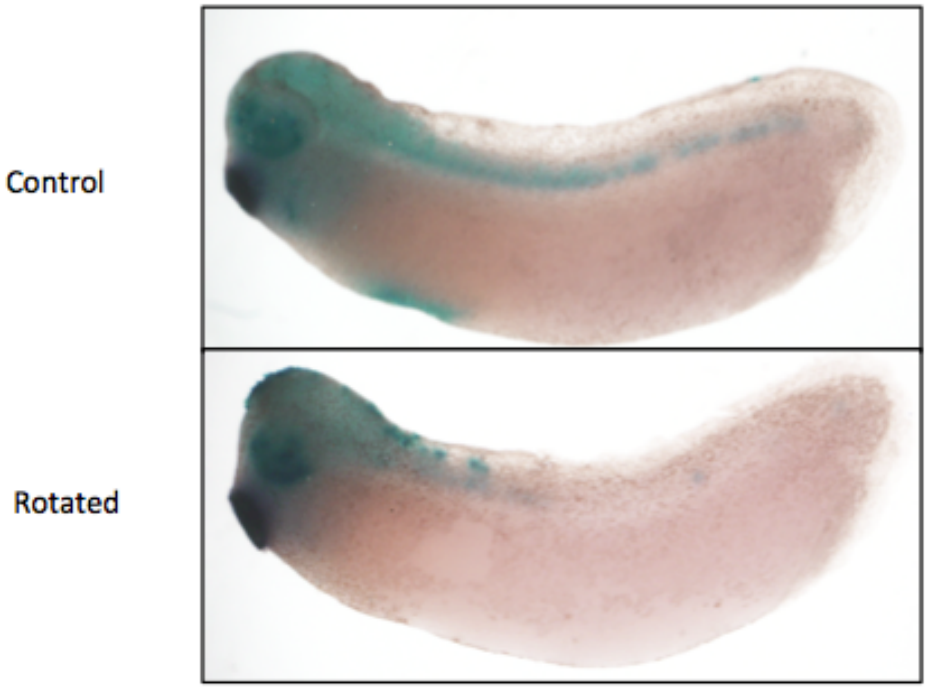

Figure 6. Donor embryos were injected with fluorescein-linked dextran and had their presumptive neural tissue removed and transplanted onto an unlabeled host at stage 11.5 (Mid-Gastrula). The tissue was either rotated relative to the host's anterior-posterior axis (Rotated) or maintained its orientation (Control). Embryos were fixed in swimming tadpole stages and assayed for *XCG-1* whole mount *in situ* hybridization using a purple precipitate NBT/BCIP reaction to visualize gene expression and afterwards using a turquoise precipitate BCIP reaction to visualize incorporation of the graft in the embryo.

Figure 7. Expression of *Otx2* in Mid-Gastrula Transplants

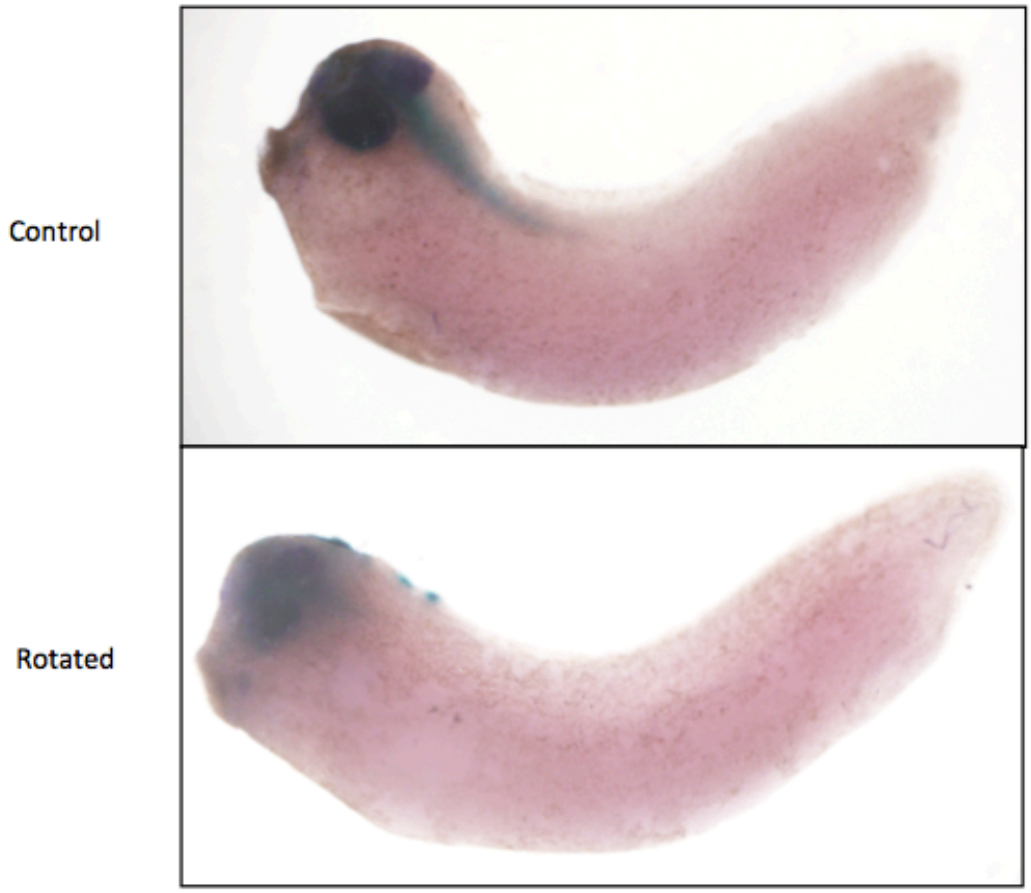

Figure 7. Donor embryos were injected with fluorescein-linked dextran and had their presumptive neural tissue removed and transplanted onto an unlabeled host at stage 11.5 (Mid-Gastrula). The tissue was either rotated relative to the host's anterior-posterior axis (Rotated) or maintained its orientation (Control). Embryos were fixed in swimming tadpole stages and assayed for *Otx2* whole mount *in situ* hybridization using a purple precipitate NBT/BCIP reaction to visualize gene expression and afterwards using a turquoise precipitate BCIP reaction to visualize incorporation of the graft in the embryo.

Figure 8. Expression of *En2* in Mid-Gastrula Transplants

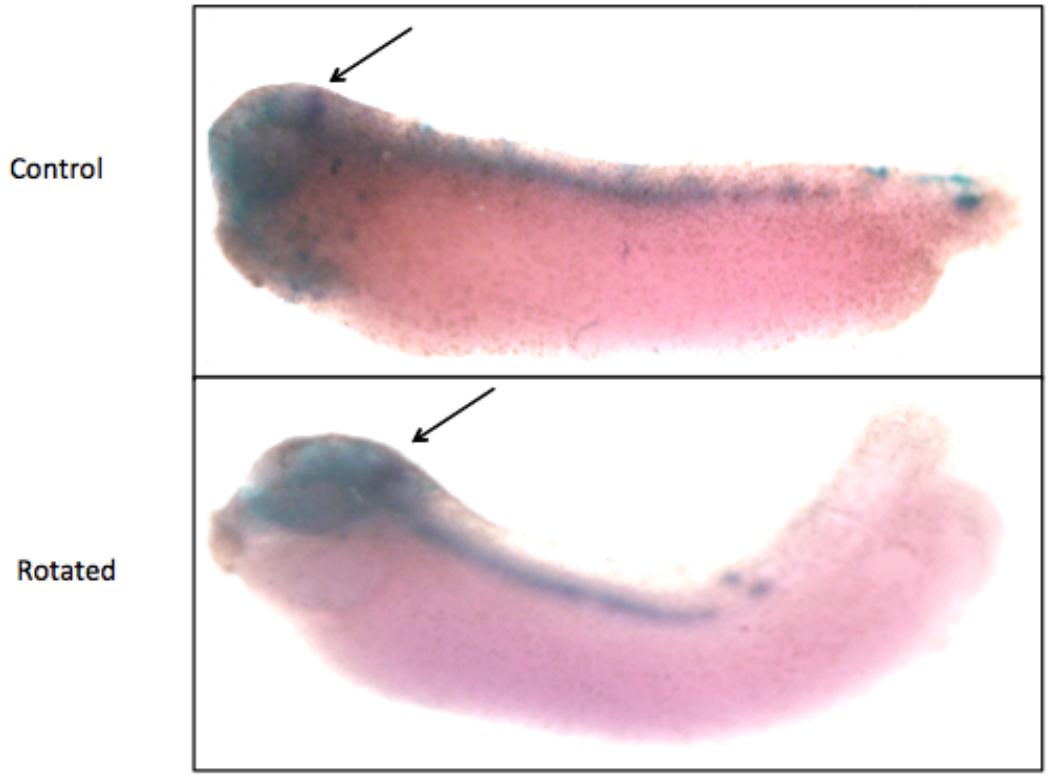

Figure 8. Donor embryos were injected with fluorescein-linked dextran and had their presumptive neural tissue removed and transplanted onto an unlabeled host at stage 11.5 (Mid-Gastrula). The tissue was either rotated relative to the host's anterior-posterior axis (Rotated) or maintained its orientation (Control). Embryos were fixed in swimming tadpole stages and assayed for *En2* whole mount *in situ* hybridization using a purple precipitate NBT/BCIP reaction to visualize gene expression and afterwards using a turquoise precipitate BCIP reaction to visualize incorporation of the graft in the embryo. Arrows were added to help visualize the purple precipitate indicative of gene expression.

Figure 9. Expression of *Krox20* in Mid-Gastrula Transplants

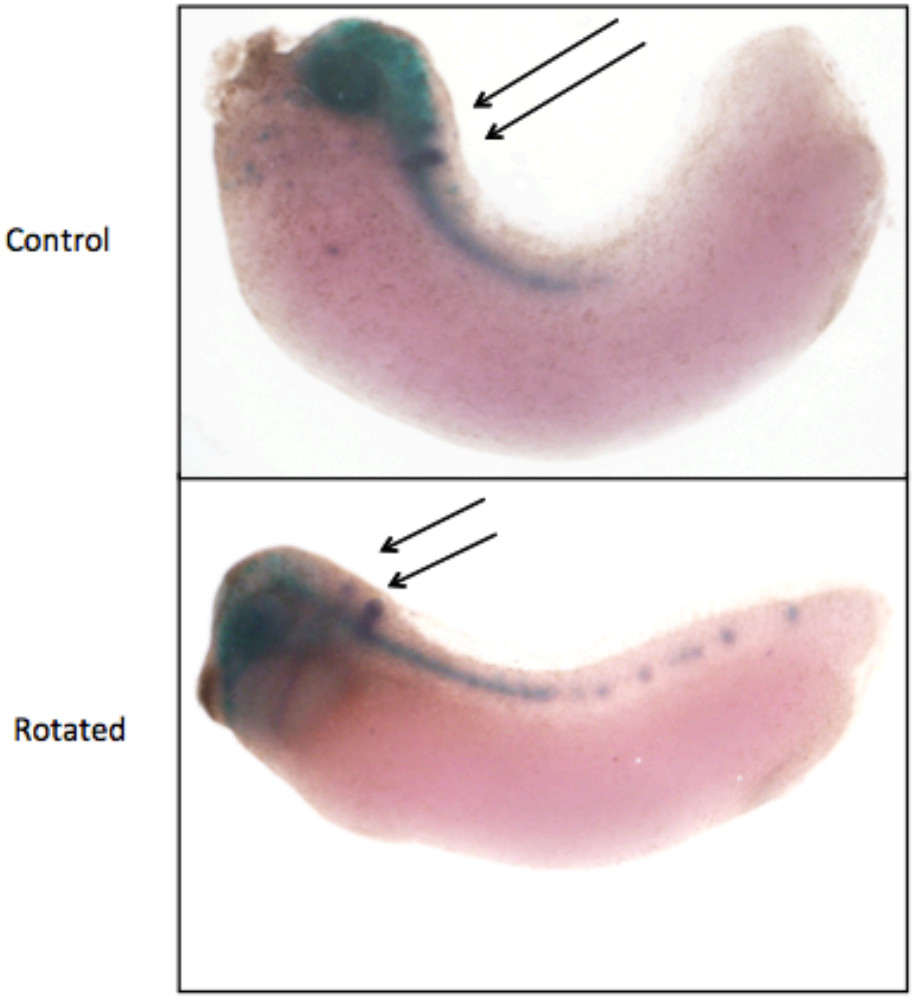

Figure 9. Donor embryos were injected with fluorescein-linked dextran and had their presumptive neural tissue removed and transplanted onto an unlabeled host at stage 11.5 (Mid-Gastrula). The tissue was either rotated relative to the host's anterior-posterior axis (Rotated) or maintained its orientation (Control). Embryos were fixed in swimming tadpole stages and assayed for *Krox20* whole mount *in situ* hybridization using a purple precipitate NBT/BCIP reaction to visualize gene expression and afterwards using a turquoise precipitate BCIP reaction to visualize incorporation of the graft in the embryo. Arrows were added to help visualize the purple precipitate indicative of gene expression.

Figure 10. Expression of Marker Genes in Late-Gastrula Rotated Transplants

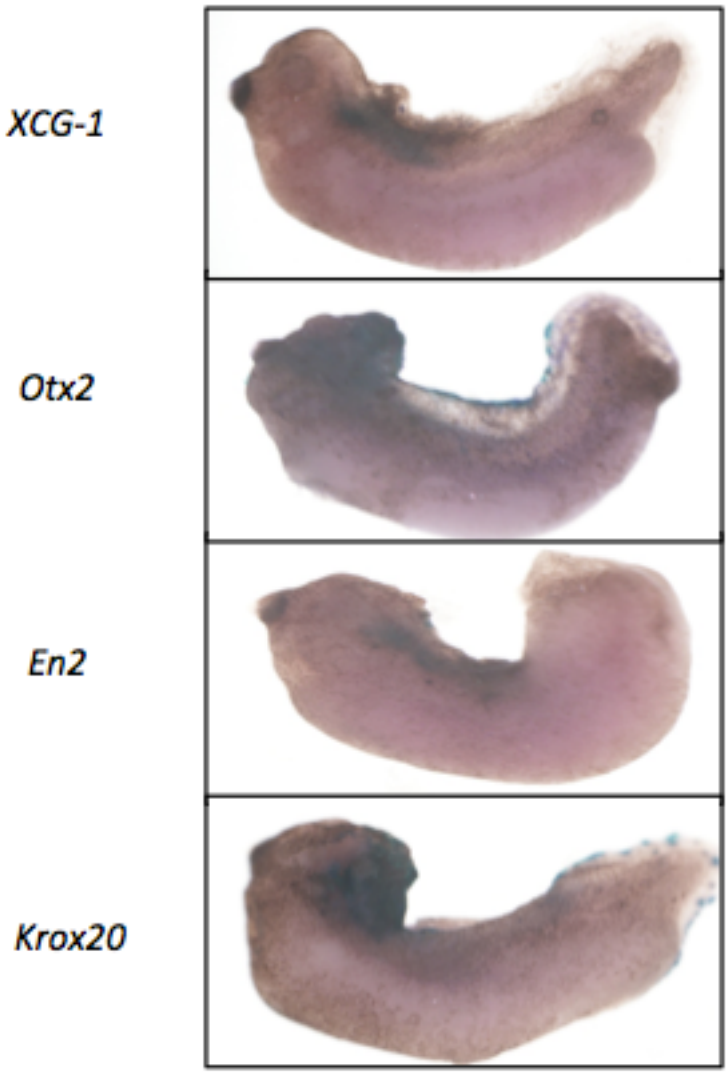

Figure 10. Donor embryos were injected with fluorescein-linked dextran and had their presumptive neural tissue removed and transplanted onto an unlabeled host at stage 12.5 (Mid-Gastrula). The tissue was rotated relative to the host's anterior-posterior axis. Embryos were fixed when un-manipulated siblings reached swimming tadpole stages and assayed for *XCG-1, Otx2, En2,* and *Krox20* expression using whole mount *in situ* hybridization using a purple precipitate NBT/BCIP reaction to visualize gene expression and afterwards using a turquoise precipitate BCIP reaction to visualize incorporation of the graft in the embryo.

6. DISCUSSION

6.1 Summary of Results

The goal of this project was to characterize the spatial and temporal bounds of neural plasticity along the anterior-posterior axis. We have determined that while the axis has been specified by stage 11.5, it remains competent to redifferentiation as evidenced by changes in gene expression in the graft to repattern itself and mimic the host-inducing environment. However, this ability is lost at the late-gastrula stage and the tissue remains incompetent to respond to the host-inducing environment drastically affecting embryonic development. This, however, is not due to a loss of healing capacity for repair, as the late-gastrula was able to incorporate tissue in the same orientation as the host's axis and recover to appear morphologically normal; in addition, the late gastrula was also able to recover post-ablation and appear morphologically normal without even replacement of the tissue.

While the whole-mount data, suggests that there is a near-complete regulation of the anterior-posterior axis following a rotation, extensive histological analysis will need to be performed in order to truly confirm that gene expression is very similar between siblings and mid-gastrula transplanted embryos. Overall, our results suggest that there is a period during gastrulation at which the anterior-posterior axis has received specification, but remains competent to redifferentiation and therefore remains plastic for a period of time. This ability is lost, however, towards the end of gastrulation even the ability for would repair still exists. Investigators performed ablations of neural tissue during neurula stages of

development and discovered doing a complete by bilateral of a region of the anterior-posterior axis resulted in a complete loss of the ablated region. However, doing unilateral ablations only on the embryos involved recovery from the contralateral tissue via proliferation (Corner, 1963). These results, in conjunction with our findings, suggest that the anterior-posterior axis has become fixed after gastrulation but maintains some regenerative capacity along the medial and lateral axis.

It seems unlikely that there is just an activation and stabilization of anterior as stated in the activation-transformation hypothesis as the posterior end of the graft is able to regulate in the anterior environment suggesting that there may be signaling that induces an anterior fate and not just a transformation event in the posterior. Thus, the adoption of the two ideas of opposing gradients and activation transformation seems most likely in accord with the resultant repatterning in the mid-gastrula transplanted tissue.

Given that the late-gastrula transplants were not able to recover when rotated but also did not show expression of the markers genes, even in an inversed fashion, suggests that an event is taking place earlier in development that post-transplantation and rotation that is not able to repattern gene expression but able to induce changes to bring about a decrease in graft gene expression. It is worthy to note, that there were fewer embryos in the 12.5 to 12.5 rotation as many did not survive to swimming tadpole stages, but because of that and due to the variability in microsurgery, more transplants will need to be done in order to fully comment on the events that are taking place that lead to the

death of the embryo and the inability to recover. What was interesting was the low survivability as opposed to hypothesized malformations, such as a cement gland in an ectopic region in the posterior. This may be due in part due to the large changes in cellular shape that occur during gastrulation as the posterior cells undergo much more extension and convergence than do the anterior neural cells (Ezin et al., 2003; Keller et al., 2008; Skoglund et al., 2008).

One portion that also has not undergone much investigation is the requirement of the mesoderm to lead to wound healing in ablations. As noted, when endoderm was exposed and the overlying mesoderm removed along with the ectoderm; the endoderm began protruding outward and leading to embryonic death.

6.2 Future Directions

While characterizing this plasticity has provided insights about the bounds at which it is able to occur, the molecular mechanisms that underlie it still remain poorly understood. Recently, membrane voltage potential has been implicated in affecting patterning along the anterior posterior axis. This voltage first becomes present at stage 12; thus it may be a key regulator of determining plasticity (Adams and Levin, 2012; Pai et al., 2015). Utilizing voltage dyes that are able to measure the membrane potential *in vivo* experiments could be designed to disrupt the endogenous membrane potential and then undergo transplantation and rotation experiments and see if the voltage affects the recovery and plasticity of the embryo(Adams and Levin, 2012).

As signaling molecules such as FGF and Wnt are involved in patterning the neural tissue and prolonged signaling events may trigger commitment to a specific regional identity and thus a loss in plasticity. As early knockdown of these genes would affect gastrulation and initial patterning, photo-morpholinos (Gene Tools) designed against a specific gene of interest and are able to control the expression of these genes in a temporal fashion. This may elucidate whether the signaling molecules that initially pattern the nervous tissue are also involved in the determination of regional identity and loss of plasticity.

Taking a more global approach, RNA-seq (Amin et al., 2014) can be performed to sequence the transcriptome of different experimental groups to get a large view of the transcription factors that are involved, This would provide some bases for candidate genes that may be involved in bringing down this plasticity or maintaining it.

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