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Anterior-Posterior Axis Plasticity in the Developing Nervous System of Xenopus laevis

Lyuba Bolkhovitinov
College of William and Mary

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

by

Lyuba Bolkhovitinov

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Williamsburg, VA
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Abstract

The establishment of the anterior-posterior (AP) axis is an essential step in the development of the central nervous system. In the model vertebrate organism *Xenopus laevis*, AP neural patterning begins during the late blastula stage and continues through gastrulation. Although the patterning of the nervous system in normal conditions has been extensively studied, less is known about how this process is able to regulate in the face of environmental perturbations. This study aims to characterize the extent and molecular basis of neural axis plasticity in *Xenopus laevis* by investigating the response of embryos to a 180-degree rotation of their AP neural axis during gastrulation. Embryos were assessed for the expression of regional marker genes using *in situ* hybridization, and also underwent global gene expression analysis using RNA-Sequencing. Our results suggest that there is a window of time between the mid- and late-gastrula stage during which embryos are able to recover from a 180-degree rotation of their neural axis and then lose this ability. At the mid-gastrula stage, embryos are able to recover from neural axis rotation and correctly express regional marker genes. By the late-gastrula stage, embryos show misregulation of regional marker genes following neural axis rotation and differential expression of genes important for neural development and patterning. Heterochronic transplants between donor and host embryos of different stages indicate that both the presumptive neural ectoderm and the underlying mesoderm play an important role in this plasticity.

Overview of the Problem

For proper embryonic development, it is essential that cells undergo determination, differentiation, patterning and morphogenesis. Another equally important aspect of organ development is the ability to respond to continual perturbations in order to adjust to changing—sometimes adverse—conditions, an ability referred to as plasticity (Skipper, Weiss, & Gray, 2010). This is particularly true of the nervous system, which is among the earliest of embryonic organ systems to develop and is required for successful development of other systems. While there has been a great deal of research into the determination and patterning of the nervous system (for a review, see Carron & Shi, 2016), relatively few molecular investigations have been conducted on plasticity. The goal of this thesis is to examine the nature of plasticity of the anterior-posterior (AP) axis, including both the extent and the molecular basis of this plasticity. We will do so using a transplant approach in which we will test the ability of the neural axis to respond to a complete AP reversal. Before presenting the details of the experiments, a brief review of the relevant literature will provide the necessary background to place these experiments in context.

Review of the Literature

Anterior-Posterior Patterning of the Neural Axis

The *Xenopus laevis* oocyte is radially symmetric, with an animal and vegetal hemisphere. Upon sperm entry into the animal hemisphere, the egg undergoes cortical rotation in which the outer cortex rotates 30° relative to the inner core in an animal-vegetal direction (Gerhart et al., 1989). After this rotation, maternal determinants deposited in the vegetal pole such as Dishevelled (Dsh) and Wnt11, are enriched on the future dorsal side of the embryo and prevent

degradation of beta-catenin by GSK3-beta in that region (Carron & Shi, 2016). The ventrally

localized maternal determinants VegT and Vg1 in combination with this stabilized beta-catenin

specify the Nieuwkoop center (NC) in the dorsal-vegetal region of the embryo, which expresses

Nodal-related genes (Smith & Harland, 1991). Distinct from the NC in the dorsal-animal region

is the blastula chordin- and noggin-expressing region (BCNE), which is required for brain

formation (Kuroda, Wessely, & De Robertis, 2004).

The NC induces the Spemann Organizer, which was discovered in Hans Spemann and

Hilde Mangold's famous 1924 experiment (Spemann & Mangold, 1924). When Spemann

transplanted the dorsal lip of an early gastrula stage embryo to the ventral side of another early-

gastrula stage embryo, the transplanted tissue induced a secondary blastopore, underwent

gastrulation, and eventually formed a full secondary body axis. This region of mesoderm is

called the Organizer for its ability to arrange a variety of tissues, including the central nervous

system. The Organizer expresses Wnt antagonists such as Cerberus, Frzb and Dickopft, and bone

morphogenic protein (BMP) antagonists such as noggin, chordin, and follistatin (De Robertis,

2006). Wnt signaling is ventralizing and posteriorizing, so inhibition of this pathway leads to

development of dorsal, anterior tissue. BMP promotes an epidermal fate, while its inhibition

causes ectoderm to develop into neural tissue. As the dorsal lip involutes during gastrulation, the

organizer moves underneath the dorsal ectoderm and releases these factors to specify neural

tissue.

The neural fate of ectoderm is stabilized by a regulatory network of transcription factors

that begin expression around the start of gastrulation. *Geminin, Sox2, Sox3, FoxD5,* and *Zic2* act

to maintain the undifferentiated state of neural progenitor cells, while *SoxD, Sox11, Zic1, Zic3,*

Xiro1, Xiro2, and *Xiro3* promote neural differentiation (Rogers, Moody, & Casey, 2009). *Sox11,*

SoxD, Zic1 and *Zic3* are induced in response to BMP antagonism, while the Iroquois genes *Xiro1-3* are strongly induced by the posteriorizing factors Wnt and FGF (Rogers et al., 2009). These genes interact with the BMP and Wnt signaling pathways to control the strength of signaling, and also regulate each other (Rogers et al., 2009).

The Spemann Organizer is a heterogenous tissue, and the timing of the involution of different cell populations helps pattern the neural axis from anterior to posterior. The first cells to involute during gastrulation are known as the head organizer; they express Wnt and BMP inhibitors and specify an anterior fate (Carron & Shi, 2016). The trunk organizer involutes next and secretes BMP antagonists, which along with active Wnt signaling promotes the formation of posterior tissues (Niehrs, 2004). This mode of patterning is described by the activation-transformation hypothesis, which proposes that the entire neural ectoderm is first "activated" to an anterior fate and then extra signaling in the posterior region "transforms" it into posterior tissue (Slack & Tannahill, 1992). More recent research has extended these views with evidence of active anteriorizing agents as well (Kiecker & Lumsden, 2012). The current hypothesis that reconciles these findings is that as neural tissue is induced it starts with an anterior character and is posteriorized, but active signaling in the anterior region is also necessary to stabilize an anterior fate (Kuroda et al., 2004).

Neural Transplantation and Competence

During the development of the nervous system, cells have been shown to have a certain amount of plasticity, or ability to change their fate in response to a changing environment (Skipper et al., 2010). This plasticity is related to the competence of cells to respond to inducing signals in their environment. When a cell is "specified" to a certain fate, it will adopt that fate when developing in isolation, but can still respond to signals and change its fate if moved to a

different region of the embryo (Slack, 1991). Once a cell has become "determined" to its fate, it will maintain this identity even in the face of inducing signals from a different region of the embryo (Slack, 1991). Thus, the time when a cell is specified but not yet determined represents a labile period in which it can respond to changes in its environment.

One of the main ways to assess the competence of cells in a developing embryo is through transplantation or explant experiments to investigate the response of cells or tissues when moved to a novel environment. Explant experiments can assess the specificity of cells by examining how they develop when isolated from their normal inducing environment. These experiments have narrowed the time frame during which the AP patterning of the neural axis is becoming specified. Saha and Grainger (1992) assayed expression of regional marker genes in neural ectoderm explants of *Xenopus laevis* and found broad, overlapping expression of anterior and posterior markers at the mid-gastrula stage that became narrow and localized by the neural plate stage. Muhr et al. (1999) identified a similar pattern of broad gene expression becoming more localized over time in gastrula stage chick embryos.

Transplanting tissue to a new environment and assessing its response can demonstrate whether or not that tissue has been determined to its fate. Transplants have been used to investigate the competence of neural ectoderm to form a variety of tissues including lens (Servetnick & Grainger, 1991), cement gland (Drysdale & Elinson, 1993), olfactory placode (Bhattacharyya & Bronner-Fraser, 2008), and even neural ectoderm (Grunz, 1990). Regarding mesodermal induction of the AP neural axis pattern, recombinations of mesoderm and neural ectoderm allow researchers to evaluate the inducing capabilities of mesoderm and competence of ectoderm from different locations in the embryo or at different time points in development. These studies have found that mesoderm has regionalized inducing capabilities (Hemmati-

Brivanlou, Stewart, & Harland, 1990) and that ectoderm loses competence to respond to those

signals by Nieuwoop and Faber stage 13-14 (Sharpe & Gurdon, 1990). Sive et al. (1990) treated

explants of *Xenopus laevis* neural ectoderm as well as whole embryos with retinoic acid (RA), an

endogenous patterning signal, and found a similar closing window of competence. By stage 15,

embryos were unable to respond to RA signaling. These studies indicate that during a period of

time prior to neurulation, cells of the neural ectoderm are not fully determined and retain

plasticity.

Neural Axis Rotation Experiments

Patterning of the anterior-posterior neural axis requires the specification of different cell

types along the axis. Assessing the plasticity of the AP axis calls for a more holistic approach in

which the ability to re-pattern the entire axis can be examined, instead of the ability of a single

cell or small piece of tissue to respond to a novel environment. To address this question,

experimenters have used reversals of the AP neural axis. If an embryo is able to recover from AP

rotation of its neural axis, the regional identities of cells along the axis are not yet fixed and the

neural tissue is able to respond to its new signaling environment. However, if the region of

transplanted tissue maintains its previous AP patterning, it was determined to its fate at the time

of transplantation.

The earliest iterations of AP axis reversal were performed by Hans Spemann (1906,

1912), who removed and rotated portions of the anterior neural plate and underlying mesoderm

(Fig. 1A). By sectioning embryos and observing their morphology, Spemann found that the

rotated piece of tissue maintained its previous identity and developed according to its prior

position in the embryo. However, the vertical signaling interactions between mesoderm and

ectoderm that influence neural patterning were not fully elucidated at this time, so the rotated

mesoderm was likely partly responsible for these results.

Roach (1945) performed axis rotation experiments on pre-neurula and neurula stage

(stage 13-14) *Ambystoma punctatum* (spotted salamander), transplanting only the ectodermal

layer from the anterior part of the neural plate (Fig. 1B). Even without the underlying mesoderm,

Roach found that the transplanted sections of tissue developed in reverse orientation, maintaining

the identity of the previous position. Nicholas (1957) performed similar axis reversals of neural

ectoderm in *Ambystoma punctatum* during the neural plate stage. He found complete recovery

after reversal, somewhat contrary to Roach's results. However, the sections of tissue used by

Nicholas were much narrower and did not cover the full expanse of the AP axis (Fig. 1C).

Sládeček (1955) performed neural axis rotations in stage 14-16 *Ambystoma mexicanum* (axolotl)

embryos and found complete regulation following rotation. Similar to Nicholas's experiment,

this result is likely explained by the fact that Sládeček's grafts were smaller than Roach's.
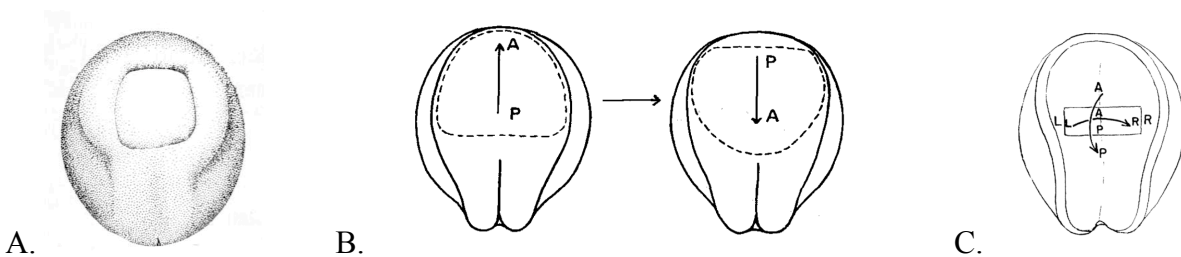
Figure 1: Schematic diagrams of early neural axis rotation experiments. 1A is from Spemann (1912), 1B is from Roach (1945) and 1C is from Nicholas (1957). All are dorsal view with anterior up and posterior down.

Jacobson (1963) performed a wide variety of rotation experiments on neural-plate stage

Ambystoma punctatum embryos, including those investigating the size of the graft and inclusion

of the neural fold. After rotation of just the neural plate (Fig. 2A), the nose, eye, and ear were

relatively normally positioned along the AP axis, although an extra eyecup formed by the ear. As

Jacobson included a greater expanse of tissue in the rotation, such as the neural plate and neural

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folds (Fig. 2B) or the neural plate, neural folds, and placodal epidermis (Fig. 2C), the

morphology of developing embryos indicated that their nervous system formed according to their

previous position in the embryo and they were unable to regulate after rotation. Jacobson

concluded that the patterning of the AP axis is relatively fixed by the neural plate stage.

A.   B.   C.
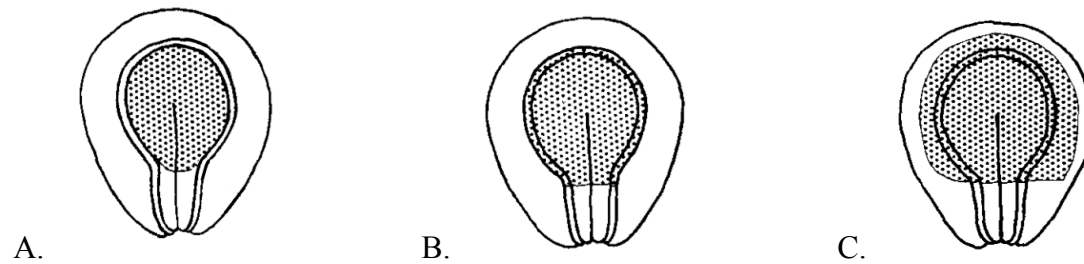
Figure 2: Schematic diagrams of neural axis rotation experiments performed by Jacobson (1963). Shaded region indicates region of neural ectoderm that was removed and rotated 180 degrees along the AP axis. 2A includes the neural plate, 2B includes the neural plate and neural folds, and 2C includes the neural plate, neural folds, and placodal epidermis. All are dorsal view with anterior up and posterior down.

Because these experiments were completed prior to the availability of molecular tools,

they relied on histology and cell shape to distinguish the regional identities of cells along the AP

axis. In addition, they did not use markers to distinguish which cells belonged to the transplanted

neural ectoderm and which cells were from the underlying host tissue. More recent techniques

provide solutions to these concerns. *In situ* hybridization can be used to label regional marker

genes along the AP axis, giving more specific information about the regional identity of tissues

to assess if an embryo has fully maintained its previous patterning or changed its fate according

to the signaling environment. Fluorescent markers can be used to distinguish donor from host

tissue and follow the fate of the transplanted tissue as development proceeds.

Experimental Overview

The present study combines classic embryological techniques with molecular markers to

investigate the plasticity of the developing AP neural axis. Based on previous research, we

hypothesized that embryos would have some amount of plasticity at stage 11.5 and would be able to recover from a 180-degree rotation of their AP neural axis, but would lose this ability by stage 12.5. At either stage 11.5 or stage 12.5, neural ectoderm was transplanted from a labeled donor embryo to a host of the same stage ("homochronic" transplant), and the piece of ectoderm was either rotated 180 degrees around the AP axis ("Rotated" transplant) or replaced in the same orientation ("Sham" transplant). Embryos were grown to either the late-neurula or hatching stage, and then were assayed for the expression of four regional marker genes expressed along the AP neural axis (*XCG-1, Otx2, En-2,* and *Krox20*). We predicted that Sham transplants and 11.5-11.5 Rotated transplants would display correct localization and expression of these regional marker genes, indicating that the transplanted tissue is able to successfully regulate in its new host environment and maintains plasticity. We predicted that 12.5-12.5 Rotated embryos would show defects in the expression of these regional marker genes, indicating a loss of plasticity and an inability to properly regulate following rotation.

The production of proper inducing signals from the mesoderm and the competence of the overlying ectoderm to respond to those signals both play a role in the patterning of the nervous system. To investigate the relative contribution of these two factors to the plasticity of the neural axis over time, a "heterochronic" transplant system was employed with donor and host embryos of different stages. Sham and Rotated transplants of neural ectoderm were performed with a stage 11.5 donor and stage 12.5 host or vice versa. The expression of the four regional marker genes was once again used to determine the extent of recovery from this perturbation. We hypothesized that the age and competence of the overlying neural ectoderm would have a greater contribution to the plasticity of the AP axis. Thus, we predicted that stage 11.5 neural ectoderm

transplanted into a stage 12.5 host would regulate better and show more correct expression of regional marker genes than stage 12.5 neural ectoderm transplanted into a stage 11.5 host.

To gain a more global insight into embryonic regulation after neural axis rotation, RNA-Sequencing analysis was performed on homochronic transplants as well as sibling embryos. We hypothesized that if embryos showed differential recovery abilities following transplantation, they would also show differential expression of a wide variety of genes. We analyzed differential gene expression between treatment groups to investigate which genes may be involved with the plasticity and repatterning of the neural axis, or which pathways may be misregulated in embryos that have lost AP axis plasticity.

Materials and Methods

Embryo Collection

Matings of adult *Xenopus laevis* were induced by injecting human chorionic gonadotropin (HCG) solution into the dorsal lymph sac using a 27½-gauge needle and a 1 ml syringe 10-12 hours before the desired time of embryo collection. Females were injected with 0.7 ml (700 U) and males with 0.5 ml (500 U) (Sive, Grainger, & Harland, 2000). Injected frogs were placed in a mating chamber with a grated opening on the bottom, which was suspended in a tank of water, allowing eggs to fall through the grate into a plastic collection tray. After the tray containing embryos was removed from the bottom of the mating tank, the water was decanted from the tray and the jelly coat was removed from the embryos using 100ml of a 2% L-cysteine solution in 0.1X Marc's Modified Ringers (MMR), with pH adjusted to 7.8-8.0 with NaOH. Embryos were rinsed three times in 0.1X MMR with 50μg/mL gentamicin to wash away excess cysteine, and then were placed in glass petri dishes containing 0.1X MMR with 50μg/mL

gentamicin at a density of 50-70 embryos per plate. Approximately 90 minutes after collection, embryo plates were sorted to remove any necrotic or improperly dividing embryos, and the solution was changed to fresh 0.1X MMR with 50 μg/mL gentamicin. Unhealthy embryos were placed in a solution of 10-15% ethanol and then discarded. If embryos were older than the neurula stage, they were first anesthetized in a solution of MS-222 (Sigma) before being moved to ethanol. Healthy embryos were stored at room temperature or in incubators kept at 14°C, 16°C, or 18°C.

Embryo Microinjection

Microinjection needles were pulled from 7" Drummond glass capillaries using a Narishige model PB-7 vertical needle puller, resulting in a needle opening diameter of 5-10μm. Needles were broken to the desired opening diameter of 20-30μm by gently pushing them through a taut KimWipe stretched over a small beaker. The diameter of the needle opening was verified using a stage micrometer.

At the 2-cell stage, *Xenopus laevis* embryos were bilaterally injected using a Drummond Nanoject 2. They were injected with 4.6nl containing 10% fluorescein-linked dextran (Fldx) into each blastomere in a dish containing 1/3X MMR with 4% ficoll (Sive et al., 2000). Injected embryos were kept in dishes containing 1/3X MMR with 4% ficoll in incubators kept at 14°C, 16°C, or 18°C. Two hours after injection, embryos were transferred to plates containing 0.1X MMR with 4% ficoll and any necrotic or improperly dividing embryos were removed. Another solution change to fresh 0.1X MMR with 4% ficoll was performed approximately six hours after the initial solution change, and unhealthy embryos were once again removed. Embryos developed overnight in the 14°C, 16°C, or 18°C incubators, and a final solution change to fresh

0.1X MMR with 4% ficoll was performed in the morning once embryos had begun to gastrulate. At this point, embryos were fluorescent-screened under a fluorescent dissecting microscope using a GFP/FITC filter to confirm successful Fldx injection. Embryos were discarded if they did not show clear fluorescent signal throughout the entirety of the embryo.

Neural Ectoderm Transplantation

Embryos were staged according to stages described by Nieuwkoop and Faber (1967). Stage 11.5 and 12.5 embryos were selected for surgery. Fldx-injected embryos were used as donors and uninjected embryos as hosts in order to delineate donor tissue from host tissue during the neural axis transplant. Homochronic transplants were performed with a matched stage donor and host, while heterochronic transplants were performed with a stage 11.5 donor and 12.5 host or vice versa. The embryos were transferred to a clay-bottomed dish with 1/3X MMR with 4% ficoll and the vitelline membrane was removed with Dumont No. 5 fine forceps. The embryos were placed snugly into wells indented into the bottom of the clay dish using dull forceps. Embryos were positioned with their dorsal side facing up, anterior pointing away from the experimenter and posterior pointing towards the experimenter. Dissections were performed using Dumont No. 5 fine forceps and needles pulled from 20μl Corning glass disposable micro-sampling pipets using a Narishige model PB-7 vertical needle puller. First, an incision was made with the needle on the posterior side of the presumptive neural ectoderm, parallel to and approximately ¼ mm above the blastopore. This initial incision was made at a depth such that it cut through the entire layer of neural ectoderm without damaging the underlying layer of mesoderm. Then, perpendicular cuts were made using the fine forceps to cut out a flap of neural ectoderm approximately 50% of the width of the embryo. The flap was carefully peeled back using the fine forceps. Care was taken to ensure that the underlying mesoderm was not damaged,

and that the piece of neural ectoderm had no mesoderm contamination. The explant was snipped off on the anterior side using fine forceps, parallel to the first incision.

The explant of neural ectoderm was first removed from the uninjected host embryo and discarded into solution. Then, the same procedure was performed on the fluorescently labeled donor embryo, but the explant was held using fine forceps and transplanted onto the open space on the dorsal side of the host embryo. During this transplantation, the explant was placed onto the host so that the explant's original AP axis orientation matched that of the host (Sham transplant) or was rotated 180° relative to the host's axis (Rotated transplant). This setup resulted in four transplant conditions for both homochronic and heterochronic transplants. For homochronic transplants the treatments were 11.5-11.5 Sham, 11.5-11.5 Rotated, 12.5-12.5 Sham, and 12.5-12.5 Rotated (Fig. 3A). For heterochronic transplants they were 11.5-12.5 Sham, 11.5-12.5 Rotated, 12.5-11.5 Sham, and 12.5-11.5 Rotated (Fig. 3B).
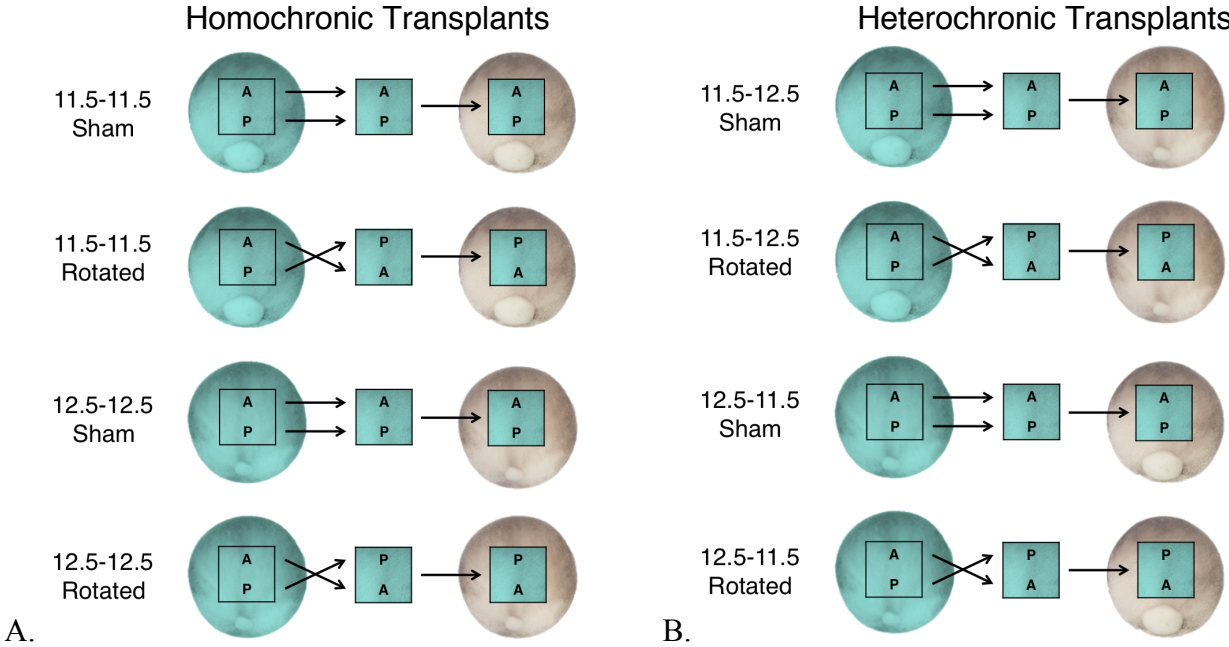

Figure 3: Diagram of transplant method. Fluorescein-injected donor embryo is on the left and uninjected host embryo is on the right. Dorsal view with anterior up and posterior down. 3A shows the four homochronic treatment groups and 3B shows the four heterochronic treatment groups.

A small glass chip made from a microscope slide coverslip was positioned to hold the transplant in place to facilitate incorporation. 2-3 hours after transplantation, the glass chip was removed and the embryos were transferred to 0.1X MMR with 4% ficoll. Embryos were allowed to grow up to stage 18 or 30, and then were imaged for both bright field and fluorescence using an Olympus SZH10 microscope with an Olympus DP71 camera or a Nikon SMZ800N microscope with a Nikon DS-Ri2 camera. The gross morphology of each embryo was observed and categorized as either normal or abnormal. Embryos classified as abnormal had underdeveloped or malformed neural features, a bent spinal cord, or a shortened body axis. After imaging, embryos were fixed in 1X MEMFA (MOPS/EGTA/Magnesium Sulfate/Formaldehyde Buffer) for subsequent analysis.

Differences in the proportion of embryos among treatment groups that were normal/abnormal/did not survive were analyzed using a chi-square test for the association between experimental treatment and morphology. The Bonferroni correction was used to correct the significance for multiple comparisons, and significance was determined at the $p < 0.05$ level. Statistical tests were run using IBM SPSS Statistics for Macintosh, Version 24.0.

Double Chromogenic in situ *Hybridization and Whole Mount Imaging*

Whole-mount in situ hybridization was performed as described by Sive et al. (2000) for the regional marker genes *XCG-1* (cement gland), *Otx2* (forebrain and eyes), *En-2* (midbrain-hindbrain boundary), and *Krox20* (rhombomeres 3 and 5 of the hindbrain). The first color reaction for the gene of interest was done with NBT/BCIP, resulting in a purple stain. After completion of the first color reaction, embryos were incubated in an anti-fluorescein alkaline phosphatase antibody to mark the location of the fluorescein-injected transplanted tissue. The second color reaction for the transplanted tissue was done with BCIP, resulting in a blue stain.

The time of fixation was determined by the strength and specificity of the signal; the reaction was terminated when non-specific background began to occur in the embryos. After development of sufficient signal, embryos were transferred to Bouin's fixative and fixed overnight at 4°C. Following fixation, embryos were transferred to bleaching solution and nutated under a fluorescent light to remove pigmentation. After bleaching was completed, embryos were transferred to 1X PBS for whole mount imaging.

Embryos were photographed for whole mount photography using either an Olympus SZH10 microscope with an Olympus DP71 camera or a Nikon SMZ800N microscope with a Nikon DS-Ri2 camera. Bright field photographs and fluorescent photographs were taken at 3X to 5.6X magnification. Whole images were globally adjusted for color, brightness, and contrast using Adobe Photoshop CS3.

Histology and Imaging of Slides

After *in situ* hybridization, embryos were dehydrated by four 15-minute washes in ethanol and 1X PBS (first with 75% 1X PBS/25% ethanol, second with 50% 1X PBS/50% ethanol, third with 25% 1X PBS/ 75% ethanol, and fourth with 100% ethanol). This was followed by three 15-minute xylene washes (first with 50% ethanol/50% xylene, second with 100% xylene, and third with 50% xylene/50% paraffin) then two two-hour paraffin incubations. Embryos were positioned in embedding boats filled with paraffin, and the paraffin was allowed to harden at room temperature for approximately 24 hours. Embryos were sectioned on a microtome into 20μm-thick frontal sections, then coverslipped and mounted on microscope slides using FlourMount. They were imaged using an Olympus MU100 camera with AmScope Imaging software. Bright field images were taken at 10X magnification and adjusted for brightness and color using Adobe Photoshop CS3.

Histology Analysis

Embryos were scored in three categories: "Old Off", "New On", and "Host On". New On refers to the extent of correct marker gene expression co-localized with transplanted neural tissue. Our definition of co-localization contained three criteria: 1) If the transplant was contiguous with the host gene expression 2) If the host gene expression was flanked by two areas of transplant on the same side 3) If the transplant was directly dorsal to the gene expression on the same side. Scores were assigned qualitatively from 0-3 to represent the extent of correct gene expression in the embryo. A score of 0 represents 0-25% of correct expression, and scores of 1, 2, and 3 represent up to 50%, 75%, or 100% of correct expression, respectively. In cases where the location of endogenous gene expression did not overlap with transplant incorporation, New On could also be scored as n/a, because the transplanted tissue did not directly express the gene of interest. In some cases, although the region of transplanted tissue did not overlap with the region of expression for the marker gene of interest, the host tissue was still able to bring up correct expression. To differentiate gene expression that was not co-localized, this was given a score from 0-3 in the category Host On. Old Off refers to the ability of an embryo to keep gene expression turned off in areas where the expected gene should not be expressed and is also scored on a qualitative scale of 0 to 3. A score of 0 indicates that the gene is not expressed in unexpected areas while a score of 3 indicates that the gene is expressed significantly in neural areas where it should not be expressed. Intermediate scores follow the same percentage guidelines as for New On.

Group differences in New On and Old Off scores were compared using Welch's ANOVA, which is robust to heterogeneity of variances. The independent variable was Treatment, with the four categories of 11.5-11.5 Sham, 11.5-11.5 Rotated, 12.5-12.5 Sham, and

12.5-12.5 Rotated for homochronics or 11.5-12.5 Sham, 11.5-12.5 Rotated, 12.5-11.5 Sham, and

12.5-11.5 Rotated for heterochronics. The dependent variable was either New On or Old Off.

Embryos fixed at stage 18 were analyzed separately from those fixed at stage 30. Scores from the

four regional marker genes were pooled and analyzed together. Post-hoc analysis was performed

using the Games-Howell test, which is also robust to heterogeneity of variances. All statistical

tests were run using IBM SPSS Statistics for Macintosh, Version 24.0.

Flash Freezing and RNA Extraction

For RNA-Sequencing analysis, homochronic transplants were performed as described

above. When a donor and host embryo were selected for transplantation, a sibling embryo of the

same stage was also selected. The sibling embryo was devitellinated but otherwise remained

unperturbed. When the sibling reached stage 18 or 30, the host and sibling embryos were imaged

and then individually flash frozen in liquid nitrogen. For single-embryo RNA extraction, a flash

frozen embryo was removed from storage in -80°C and 300µl TRIzol was added to the tube. The

embryo was homogenized with a pestle until it was completely dispersed into the TRIzol. The

sample was vortexed for 15 seconds and then incubated at room temperature for 5 minutes. The

sample was centrifuged for 15 seconds, then 60µl chloroform was added to the sample and it was

vortexed again for 15 seconds. The sample was incubated at room temperature for 3 minutes, and

then the entire contents of the tube were transferred to a Phase Lock Gel Heavy tube (Quantabio)

to help separate the aqueous and organic phases. Phase Lock Gel Heavy tubes were centrifuged

for 30 seconds at $12,000 \times g$ before this step in order to collect all gel at the bottom of the tube.

After the sample was added to the Phase Lock Gel Heavy tube, it was centrifuged for 15 min at

$12,000 \times g$ at 4°C. After the spin, the aqueous layer was loaded into the MagMAX™ Express-96

Standard Magnetic Particle Processor and processed using the MagMAX™-96 Total RNA

Isolation Kit. RNA was eluted in Elution Buffer and stored at -80°C. RNA was obtained from embryos in ten experimental categories: 11.5-11.5 Sham, 11.5-11.5 Rotated, 12.5-12.5 Sham, 12.5-12.5 Rotated, and Sibling at Stage 18 and Stage 30, with a total of n=5 embryos for each category.

RNA-Sequencing Analysis

RNA was sent to the Oklahoma Medical Research Foundation for cDNA synthesis and RNA-Sequencing. Each sample returned a read depth of 30-40 million paired-end reads. Reads were mapped to the *Xenopus laevis* reference genome Version 9.1 using HiSat2 (Kim, Langmead, & Salzberg, 2015). The number of reads aligning to each specific gene was determined using HTSeq-Count (Anders, Pyl, & Huber, 2015), along with the genome annotation XENLA_UTAmayball (Taejoon Lab, Ulsan National Institute of Science and Technology, Republic of Korea) to provide information on gene loci and exon boundaries. Finally, differential read counts between conditions were analyzed using DESeq2 (Love, Huber, & Anders, 2014). The Bonferroni correction was used to correct for multiple comparisons, and genes were ranked by adjusted p value. Significance was determined at the $p<0.05$ level.

Results

Homochronic Transplants

Morphology

In order to determine the overall viability and level of morphological impairment of the embryos following neural ectoderm transplantation, we analyzed the gross morphology of embryos grown up to the late-neurula (Fig. 4) or hatching stage (Fig. 5).
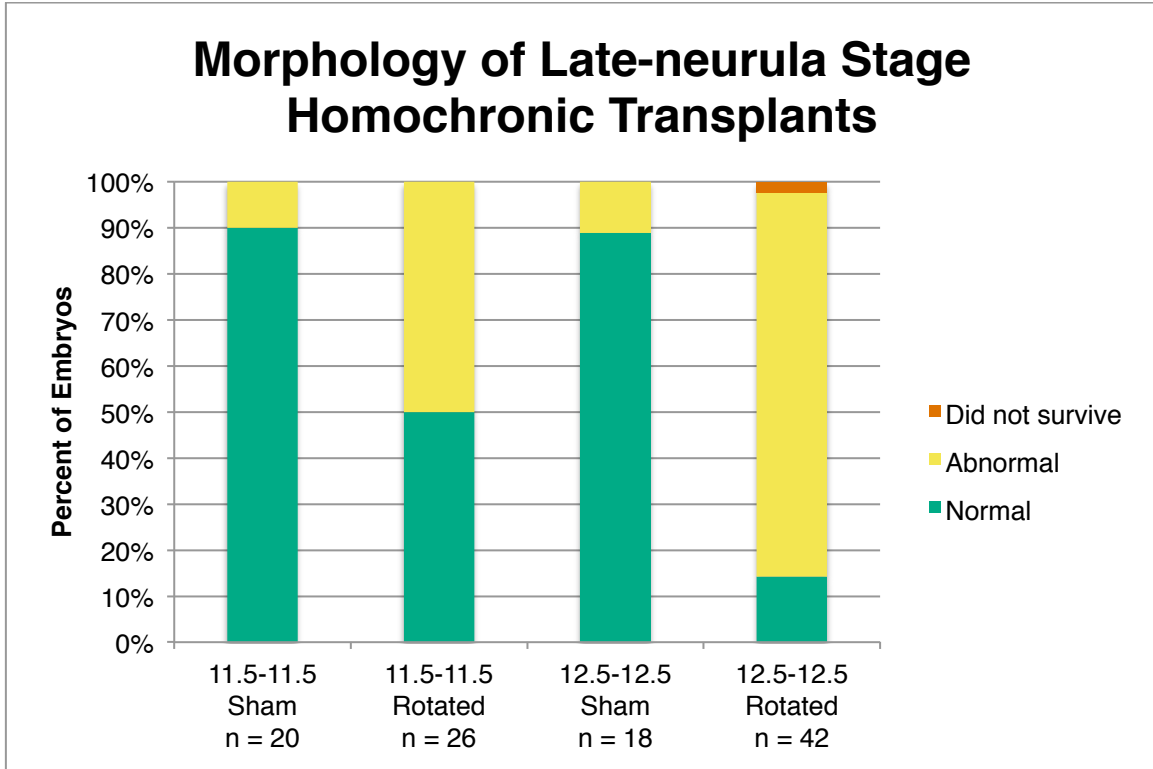
Figure 4: Survivability and morphology of homochronic transplants at late-neurula stage.
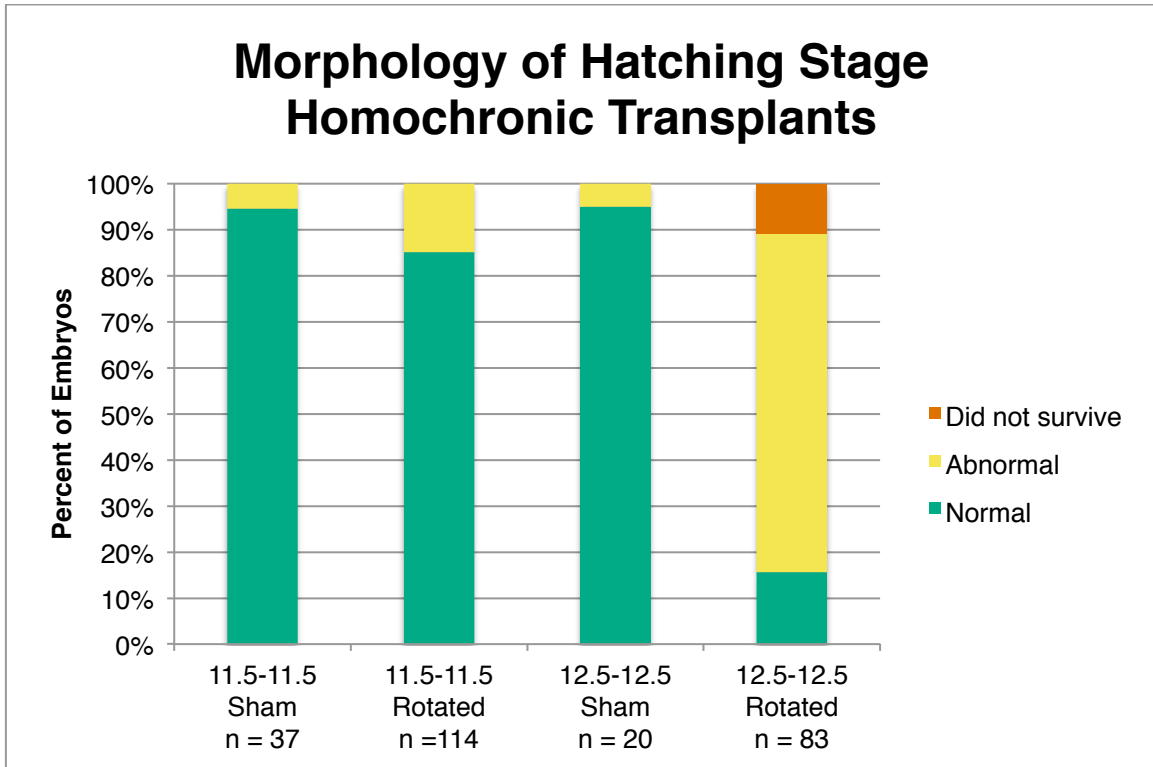

Figure 5: Survivability and morphology of homochronic transplants at hatching stage.

At the late-neurula stage, Sham embryos from transplants performed at both stage 11.5 and 12.5 develop normally almost 90% of the time. They both have significantly higher proportions of normally developing embryos than the population distribution (p = 8.6E-4 for 11.5-11.5 Sham, p = 3.5E-3 for 12.5-12.5 Sham). About 50% of 11.5-11.5 Rotated embryos develop normally, compared to only 15% of 12.5-12.5 Rotated embryos. The proportion of abnormally developing 12.5-12.5 Rotated embryos is highly significantly different from the population distribution (p = 3.1E-8).

At the hatching stage, Sham transplants show an even higher proportion of normal development, with over 90% of Sham embryos developing normally. 11.5-11.5 Rotated embryos show increasingly normal development at the hatching stage, with around 85% of embryos developing normally. All three of these groups have significantly higher proportions of normal development than the population distribution (p = 4.3E-4 for 11.5-11.5 Sham, p = 8.2E-9 for 11.5-11.5 Rotated, p = 0.036 for 12.5-12.5 Sham). In contrast, 12.5-12.5 Rotated Embryos have a highly significant increase in proportion of abnormally developing embryos (p = 0.00).

These results suggest that there is not a loss in overall healing ability as embryos develop from stage 11.5 to stage 12.5, because Sham transplants at both stages largely develop normally. At stage 11.5, embryos are able to recover following neural axis rotation, indicating that the transplanted neural tissue is able to correctly respecify and adopt the fate of its new host environment. In contrast, embryos are not able to recover following neural axis rotation at stage 12.5, indicating a loss in plasticity of the neural axis.

Regional Marker Gene Expression

In order to further investigate the plasticity of the AP axis at the molecular level, *in situ* hybridization was performed for four regional marker genes (*XCG-1, Otx2, En-2, and Krox20*).

The endogenous expression of each of these genes is as follows: *XCG-1* in the cement gland, *Otx2* in the eye and forebrain, *En-2* in the midbrain, and *Krox20* in rhombomeres 3 and 5 of the hindbrain. Embryos were also incubated with anti-fluor alkaline phosphatase antibody to determine the presence and extent of the transplanted donor tissue. This approach resulted in two distinct colors of staining in the processed embryo: purple for gene expression and blue for transplanted tissue (Figs. 6 and 7).
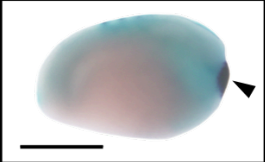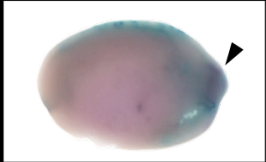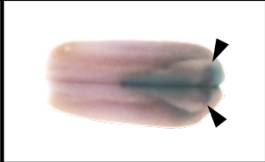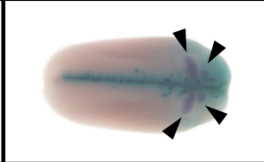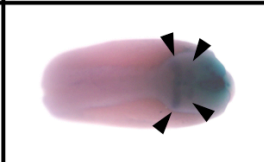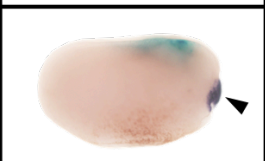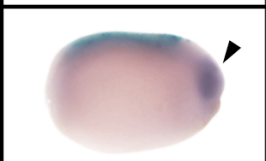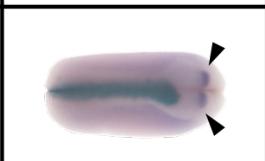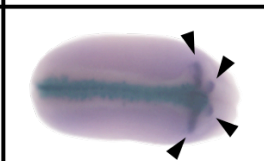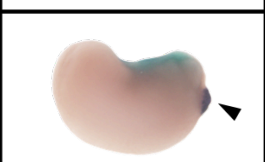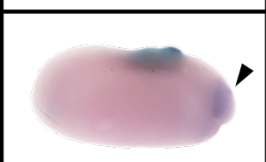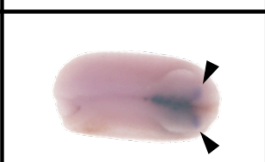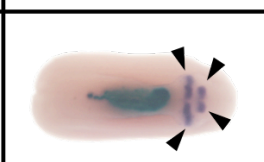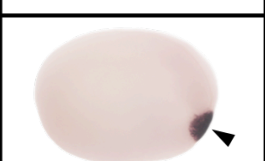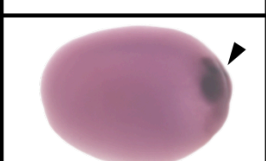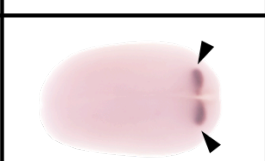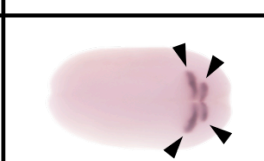
Late-neurula	*XCG-1*	*Otx2*	*En-2*	*Krox20*
11.5-11.5 Sham				
11.5-11.5 Rotated				
12.5-12.5 Sham				
12.5-12.5 Rotated				
Sibling				

Figure 6: Representative examples of expression of regional marker genes in homochronic transplants at late-neurula stage. Lateral view with dorsal facing up for *XCG-1* and *Otx2*, and dorsal view for *En-2* and *Krox20*. Anterior is to the right in all images. Purple stain marked with an arrow indicates gene expression and blue stain indicates transplant incorporation. Scale bar represents 1mm.
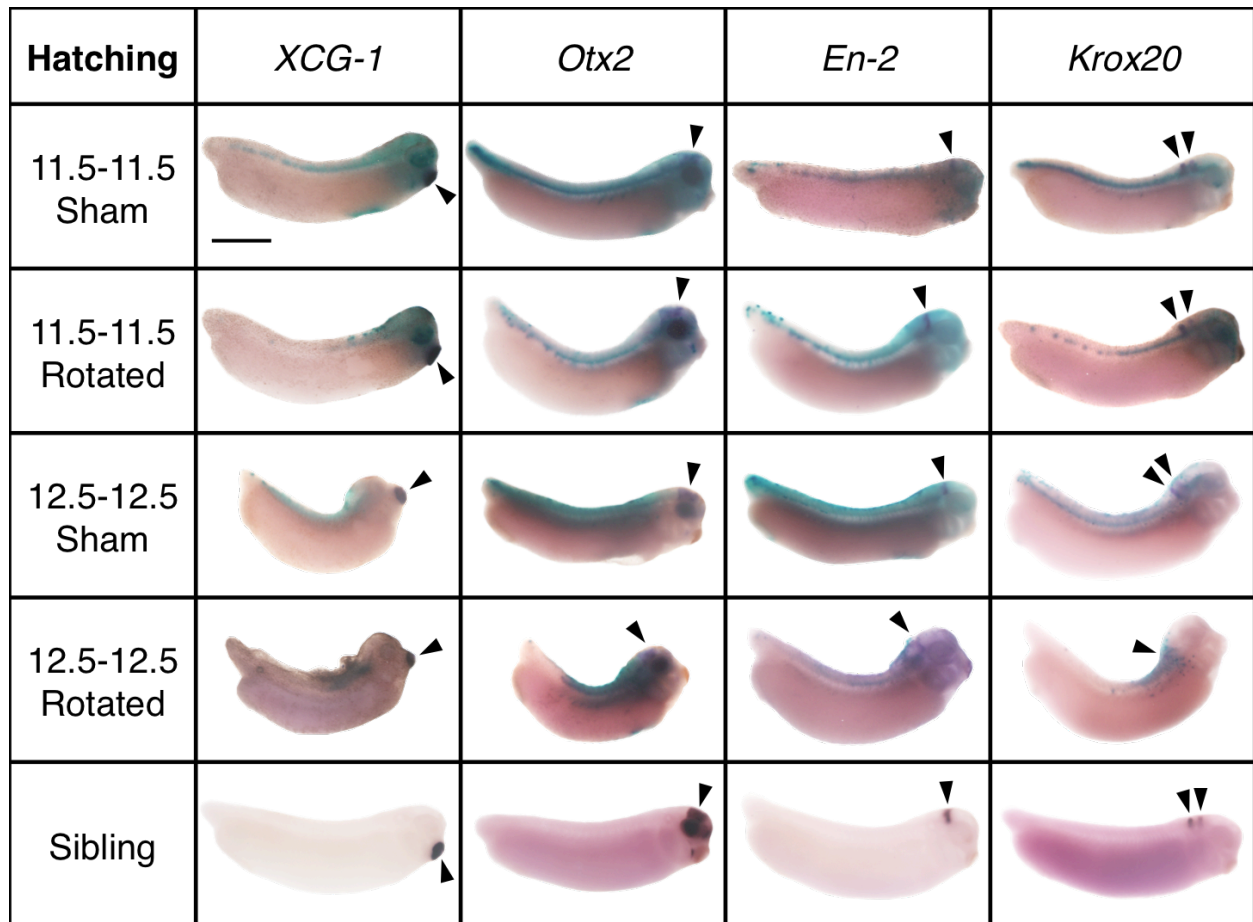
Figure 7: Representative examples of expression of regional marker genes in homochronic transplants at hatching stage. Lateral view of all embryos with dorsal facing up; anterior is to the right. Purple stain marked with an arrow indicates gene expression and blue stain indicates transplant incorporation. Scale bar represents 1mm.

In order to examine gene expression with greater resolution, embryos were embedded in paraffin and sectioned, and then each of the histological sections was imaged (Figs. 8 and 9). Each embryo was scored in three categories: New On, Old Off, and Host On (Figs. 10 and 11). New On refers to the ability of the transplanted tissue to correctly bring up marker gene expression in its new location in the host embryo. A score of 0 represents 0-25% of correct expression, and scores of 1, 2, and 3 represent up to 50%, 75%, or 100% of correct expression, respectively. A score of "n/a" was given if the transplanted tissue did not incorporate in the location of endogenous gene expression for the particular marker gene. Old Off refers to the

ability of the transplanted tissue to suppress ectopic gene expression in the region where it used to be. A score of 0 indicates that no ectopic gene expression was co-localized with transplanted tissue, while a score of 3 indicates large amounts of aberrant gene expression co-localized with the transplant. Finally, Host On was used to describe situations when the host tissue brought up gene expression without co-localization with transplanted tissue. An embryo with perfect regulation of its AP axis would have a score of 3 for New On and a score of 0 for Old Off.

Late-neurula	XCG-1		Otx2		En-2		Krox20	
	New On	Old Off	New On	Old Off	New On	Old Off	New On	Old Off
11.5-11.5 Sham								
11.5-11.5 Rotated								
12.5-12.5 Sham								
12.5-12.5 Rotated								

Figure 8: Representative histology for homochronic transplants at late-neurula stage. Dorsal is facing up. Purple stain indicates gene expression and blue stain indicates transplant incorporation. Scale bar represents 1 mm.

| Hatching	XCG-1		Otx2		En-2		Krox20	
	New On	Old Off	New On	Old Off	New On	Old Off	New On	Old Off
11.5-11.5 Sham								
11.5-11.5 Rotated								
12.5-12.5 Sham								
12.5-12.5 Rotated								

Figure 9: Representative histology for homochronic transplants at hatching stage. Dorsal is facing up. Purple stain indicates gene expression and blue stain indicates transplant incorporation. Scale bar represents 1 mm.
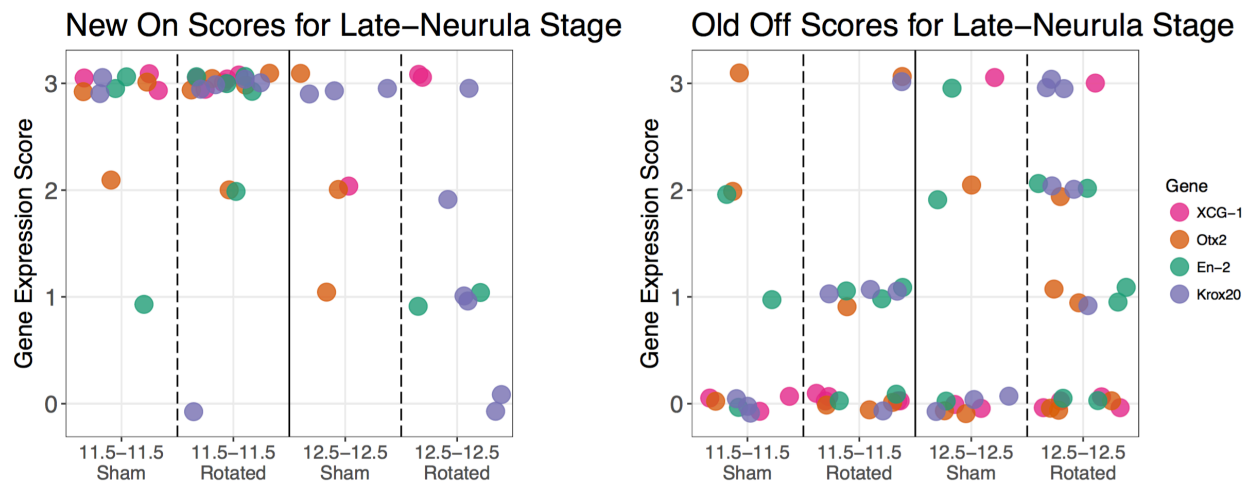
Figure 10: Histology scores for homochronic transplants at late-neurula stage.
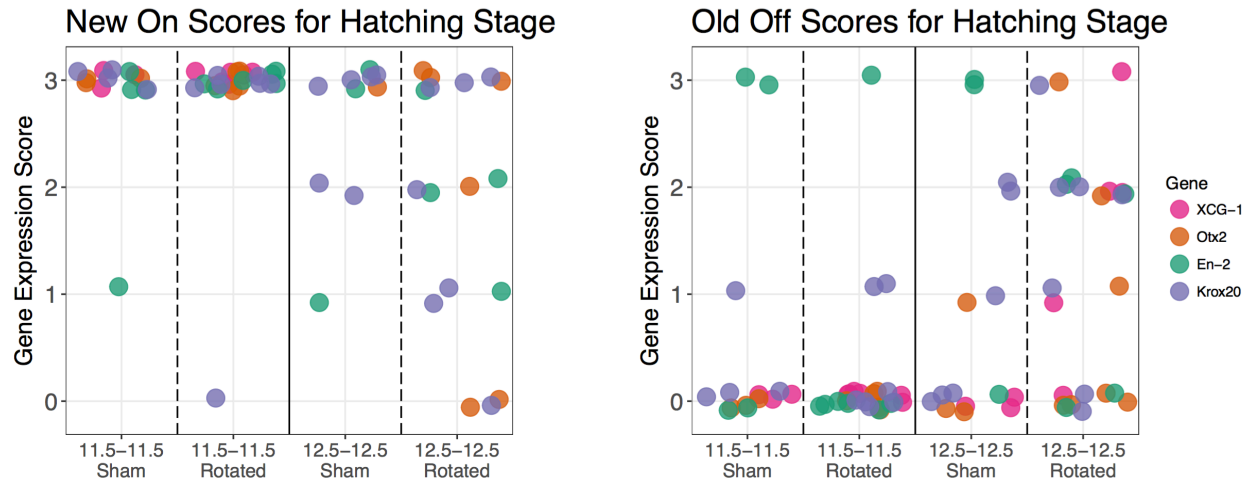
30

Figure 11: Histology scores for homochronic transplants at hatching stage.

Homochronic Stage 18 (Neural Tube Stage)

By stage 18, transplants from the four experimental conditions differed in their ability to correctly express regional marker genes (Welch's ANOVA for New On, $p = 0.048$). Post-hoc analysis revealed that 12.5-12.5 Rotated embryos had significantly lower scores for New On than 11.5-11.5 Sham embryos ($p = 0.050$) and 11.5-11.5 Rotated embryos ($p = 0.040$). 12.5-12.5 Rotated embryos also had lower New On scores than 12.5-12.5 Sham embryos, although this difference was not statistically significant ($p = 0.251$). No other between-group comparisons were statistically significant. These results indicate that at Stage 18, 12.5-12.5 Rotated embryos have a decreased ability to correctly bring up regional marker gene expression, indicating that they are not able to successfully re-pattern their neural axis following inversion at stage 12.5. 11.5-11.5 Rotated transplants did not have significantly different New On scores compared to Sham transplants ($p = 1.00$), suggesting that embryos are able to correctly regulate expression of regional marker genes following neural axis inversion at stage 11.5. Old Off scores, indicating the amount of ectopic regional marker gene expression, did not significantly differ among treatment groups (Welch's ANOVA for Old Off, $p = 0.417$).

Homochronic Stage 30 (Hatching Stage)

At stage 30, there were also statistically significant differences between treatment groups in their ability to correctly express regional marker genes (Welch's ANOVA for New On, p = 0.025). Post-hoc analysis indicated that the same pairwise differences as found at stage 18 were contributing to this difference at stage 30. 12.5-12.5 Rotated embryos had significantly lower scores for New On than 11.5-11.5 Sham embryos (p = 0.025) and 11.5-11.5 Rotated embryos (p = 0.019). 12.5-12.5 Rotated embryos also had lower New On scores than 12.5-12.5 Sham embryos, although this difference was not statistically significant (p = 0.215). No other between-group comparisons were significant. 12.5-12.5 Rotated embryos persist in their inability to correctly express regional marker genes as development proceeds; they do not show statistically significant increasing compensation for the perturbation over time. In fact, the differences between the New On scores of 12.5-12.5 Rotated embryos and the other groups are even more significant at stage 30 than at stage 18, suggesting that the initial misregulation in these embryos could have worsening effects later in development.

Hatching stage embryos also showed significant differences in the amount of ectopic gene expression, as measured by Old Off score (Welch's ANOVA for Old Off, p = 0.003). Post-hoc analysis indicated that the only significant pairwise difference was between 12.5-12.5 Rotated and 11-5-11.5 Rotated embryos, with 12.5-12.5 Rotated embryos having significantly higher Old Off scores (p = 0.002). In addition to 12.5-12.5 Rotated embryos not bringing up regional marker gene expression in the correct location, they also tend to have increased ectopic expression. For both New On and Old Off scores, 11.5-11.5 Rotated embryos are not significantly different from Sham transplant embryos (p = 1.00 for New On, p = 0.784 for Old

Off), indicating that they are similarly able to recover following rotation of their anterior-posterior neural axis.

These results agree with the initial morphological classification of transplant embryos in suggesting that embryos lose the ability to recover from anterior-posterior neural axis rotation by stage 12.5. However, embryos still show plasticity following this perturbation if it is performed at stage 11.5. Thus, the time between stage 11.5 and 12.5 represents a window of neural axis plasticity during which embryos progressively lose the ability to re-pattern the AP axis following inversion.

Heterochronic Transplants

Morphology

The closing of this window of neural axis plasticity between stage 11.5 and stage 12.5 could be due to a loss of competency of the overlying neural ectoderm, or a loss in inducing signal from the underlying mesoderm. In order to elucidate this difference, heterochronic transplants were performed with a stage 11.5 donor and stage 12.5 host or vice versa. The four experimental treatments were 11.5-12.5 Sham, 11.5-12.5 Rotated, 12.5-11.5 Sham, and 12.5-11.5 Rotated. In order to determine the overall viability and level of morphological impairment of the embryos following neural ectoderm transplantation, we analyzed the gross morphology of embryos grown up to the late-neurula (Fig. 12) or hatching stage (Fig. 13).
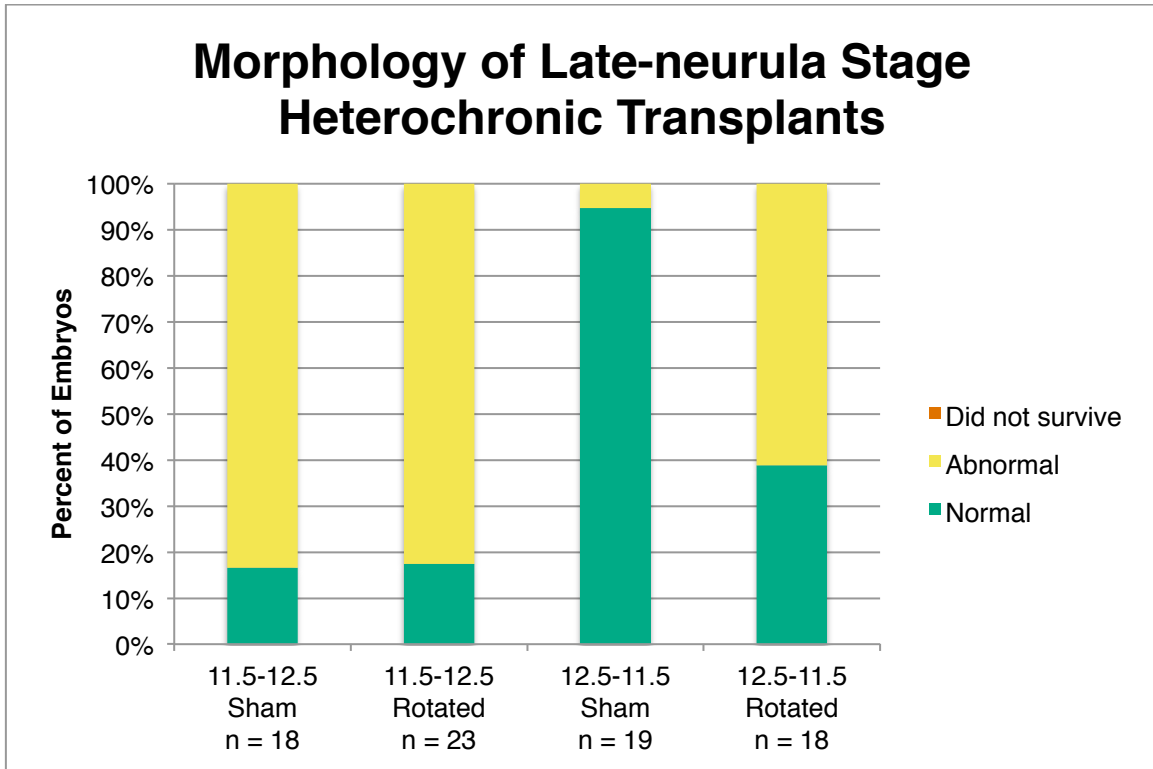
Figure 12: Survivability and morphology of heterochronic transplants at late-neurula stage.
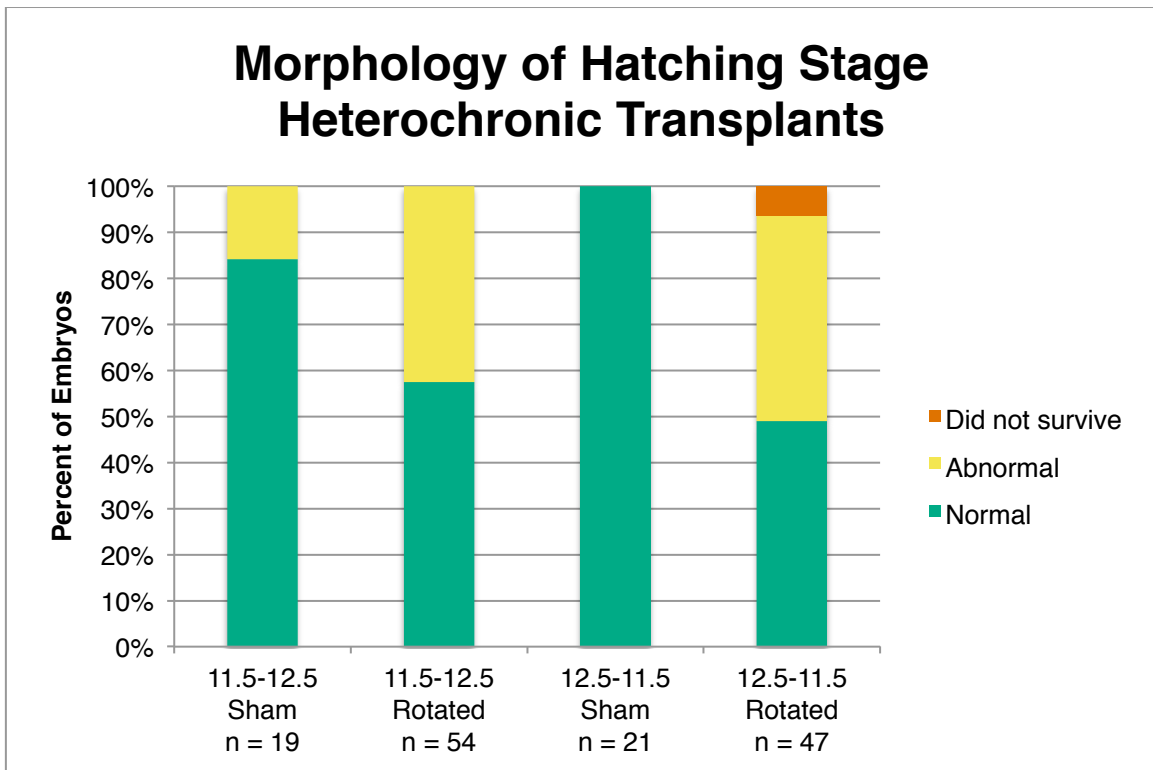

Figure 13: Survivability and morphology of heterochronic transplants at hatching stage.

At the late-neurula stage, all categories of transplant show fairly high levels of abnormal development except for 12.5-11.5 Sham embryos, which have statistically significantly lower levels of abnormal expression (p = 3.5E-7). By the hatching stage, 12.5-11.5 Sham embryos are still the most likely to develop normally, but by a smaller margin. Their proportion of normal development is still significantly higher than the population distribution (p = 2.8E-3). At the hatching stage, both categories of Rotated transplants have a higher incidence of abnormal development than either category of Sham transplant. Although 12.5-11.5 Rotated embryos have a slightly higher percentage of abnormal development and some embryos that did not survive to the hatching stage, there is still not a statistically significant difference between neural axis rotation with a mid-gastrula donor and late-gastrula host or vice versa. These results do not definitively indicate whether the overlying ectoderm or underlying mesoderm plays the greater role in determining neural axis plasticity, and suggests that both are involved.

Regional Marker Gene Expression

In order to more precisely investigate the patterning of the neural axis at the molecular level, *in situ* hybridization was performed for the same four regional marker genes as for the homochronic embryos (*XCG-1, Otx2, En-2,* and *Krox20*) (Figs. 14 and 15).
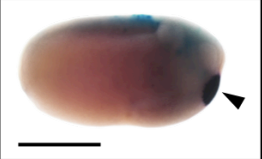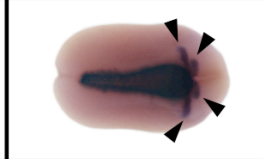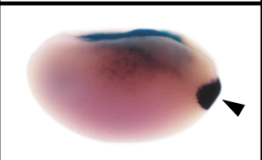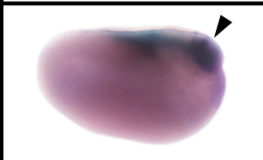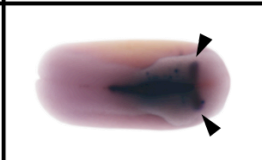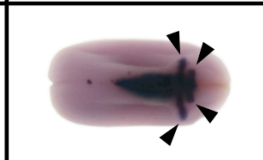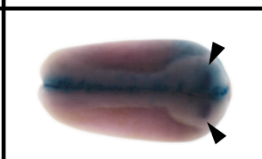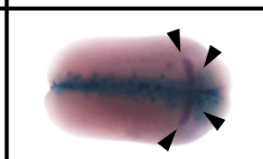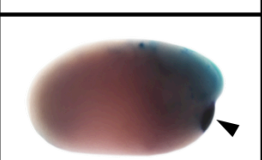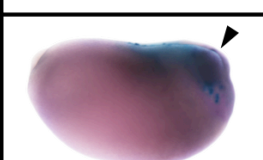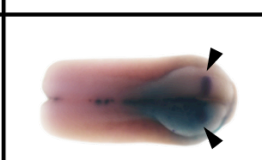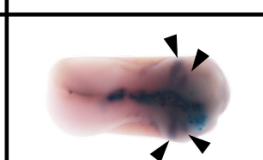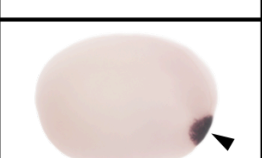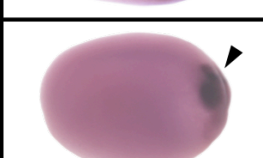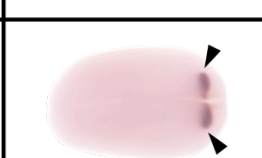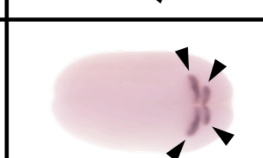
Late-neurula	XCG-1	Otx2	En-2	Krox20
11.5-12.5 Sham				
11.5-12.5 Rotated				
12.5-11.5 Sham				
12.5-11.5 Rotated				
Sibling				

Figure 14: Representative examples of expression of regional marker genes in heterochronic transplants at late-neurula stage. Lateral view with dorsal facing up for *XCG-1* and *Otx2*, and dorsal view for *En-2* and *Krox20*. Anterior is to the right in all images. Purple stain marked with an arrow indicates gene expression and blue stain indicates transplant incorporation. Scale bar represents 1mm.
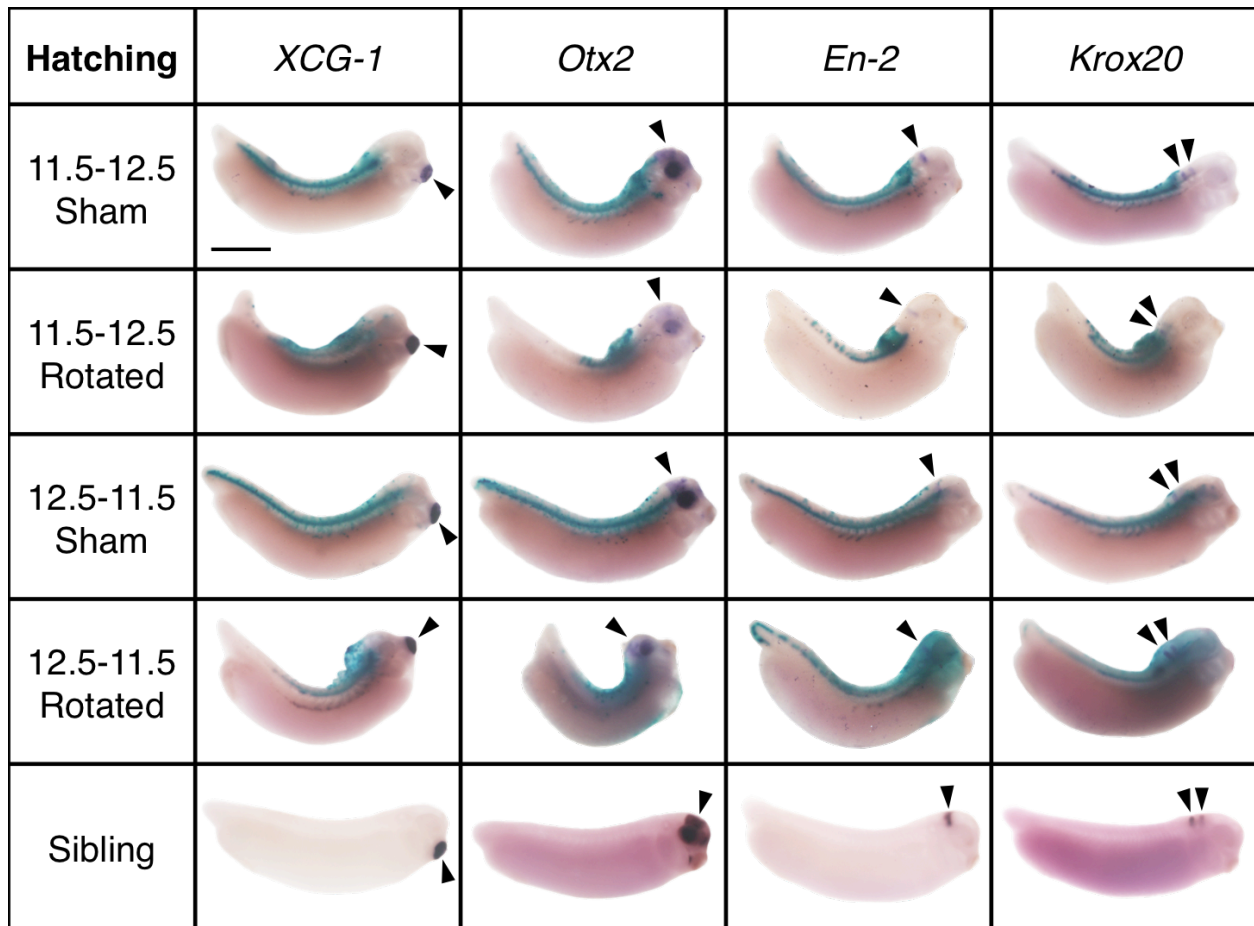
Figure 15: Representative examples of expression of regional marker genes in heterochronic transplants at hatching stage. Lateral view of all embryos with dorsal facing up; anterior is to the right. Purple stain marked with an arrow indicates gene expression and blue stain indicates transplant incorporation. Scale bar represents 1mm.

In order to examine gene expression with greater resolution, embryos were embedded in paraffin and sectioned, and then each of the histological sections was imaged (Figs. 16 and 17). Each embryo was scored in three categories: New On, Old Off, and Host On (Figs. 18 and 19). New On refers to the ability of the transplanted tissue to correctly bring up marker gene expression in its new location in the host embryo. A score of 0 represents 0-25% of correct expression, and scores of 1, 2, and 3 represent up to 50%, 75%, or 100% of correct expression, respectively. A score of "n/a" was given if the transplanted tissue did not incorporate in the location of endogenous gene expression for the particular marker gene. Old Off refers to the

ability of the transplanted tissue to suppress ectopic gene expression in the region where it used to be. A score of 0 indicates that no ectopic gene expression was co-localized with transplanted tissue, while a score of 3 indicates large amounts of aberrant gene expression co-localized with the transplant. Finally, Host On was used to describe situations when the host tissue brought up gene expression without co-localization with transplanted tissue. An embryo with perfect regulation of its AP axis would have a score of 3 for New On and a score of 0 for Old Off.

Late-neurula	XCG-1		Otx2		En-2		Krox20	
	New On	Old Off	New On	Old Off	New On	Old Off	New On	Old Off
11.5-12.5 Sham								
11.5-12.5 Rotated								
12.5-11.5 Sham								
12.5-11.5 Rotated								

Figure 16: Representative histology for heterochronic transplants at late-neurula stage. Dorsal is facing up. Purple stain indicates gene expression and blue stain indicates transplant incorporation. Scale bar represents 1mm.

Hatching	XCG-1		Otx2		En-2		Krox20	
	New On	Old Off	New On	Old Off	New On	Old Off	New On	Old Off
11.5-12.5 Sham								
11.5-12.5 Rotated								
12.5-11.5 Sham								
12.5-11.5 Rotated								

Figure 17: Representative histology for heterochronic transplants at hatching stage. Dorsal is facing up. Purple stain indicates gene expression and blue stain indicates transplant incorporation. Scale bar represents 1mm.

Figure 18: Histology scores for heterochronic embryos at late-neurula stage.
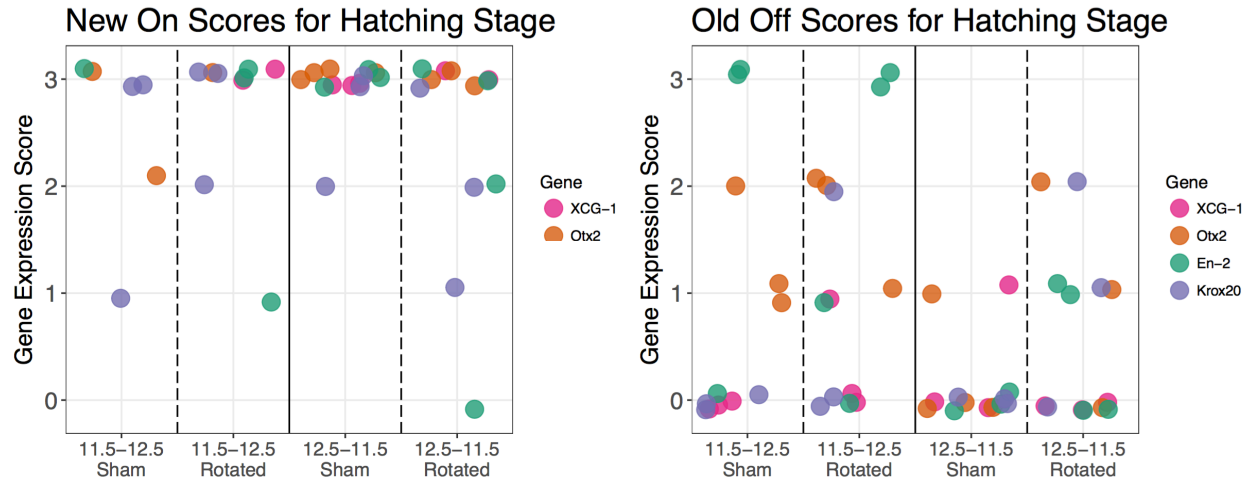
Figure 19: Histology scores for heterochronic embryos at hatching stage.

Heterochronic Stage 18 (Neural Tube Stage)

At stage 18, embryos from the four heterochronic treatment groups did not significantly differ in their ability to correctly express regional marker genes (Welch's ANOVA for New On, p = 0.170). However, there were highly significant differences among the groups in their levels of ectopic regional marker gene expression (Welch's ANOVA for Old Off, p = 0.000). Post-hoc analysis revealed that 12.5-11.5 Sham embryos had significantly lower levels of ectopic gene expression than both 11.5-12.5 Rotated embryos (p = 0.001) and 12.5-11.5 Rotated embryos (p = 0.005). 12.5-11.5 Sham embryos also had lower Old Off scores than 11.5-12.5 Sham embryos that were trending towards significance (p = 0.100). At stage 18, there is no clear difference between the ability of embryos to re-pattern their neural axis following rotation at stage 11.5 versus 12.5.

Heterochronic Stage 30 (Hatching Stage)

At stage 30, there were also no significant differences among heterochronic treatment groups in their levels of correct regional marker gene expression. (Welch's ANOVA for New On, p = 0.170). However, there were differences among the groups in their levels of ectopic regional marker gene expression as at stage 18 (Welch's ANOVA for Old Off, p = 0.016). Post-hoc analysis indicated that the only significant pairwise difference was that 12.5-11.5 Sham embryos had significantly lower Old Off scores than 11.5-12.5 Rotated embryos (p = 0.041). It is difficult to interpret this difference because 12.5-11.5 Sham embryos and 11.5-12.5 Rotated embryos differ on two dimensions. However, it seems that at both stage 18 and stage 30, 12-5.11.5 Sham embryos have the least ectopic expression of regional marker genes. This result is consistent with the morphology classification of heterochronic transplants, which indicated that 12.5-11.5 Sham embryos had the highest percentage of embryos with normal morphology out of all the groups. Based on these results, no clear distinction can be made between 11.5-12.5 Rotated and 12.5-11.5 Rotated embryos.

RNA-Sequencing

To investigate differential gene expression between transplant conditions, homochronic transplants were performed as described above. At the same time as donor and host embryos were selected for transplantation, a sibling embryo of the same stage was selected and devitellinated. When the sibling embryo reached either the late-neurula or hatching stage, the host and the sibling were imaged. The host embryo was imaged with both bright field and fluorescence to confirm incorporation of the transplant (Figs. 20 and 21). Then, embryos were

individually flash frozen in liquid nitrogen. RNA was extracted from single embryos, and then

RNA-Sequencing was performed on n=5 embryos from each category.
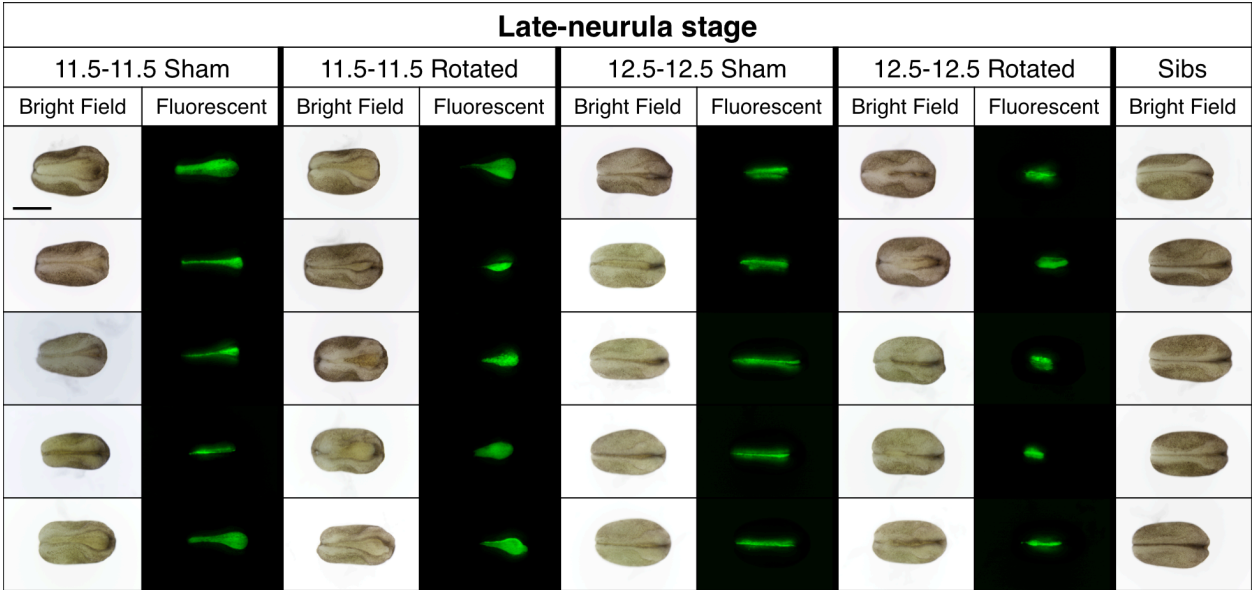
Figure 20: Late-neurula stage embryos used for RNA-Sequencing analysis. Fluorescent image indicates location or transplant incorporation. Dorsal view of all embryos; anterior is to the right. Scale bar represents 1mm.
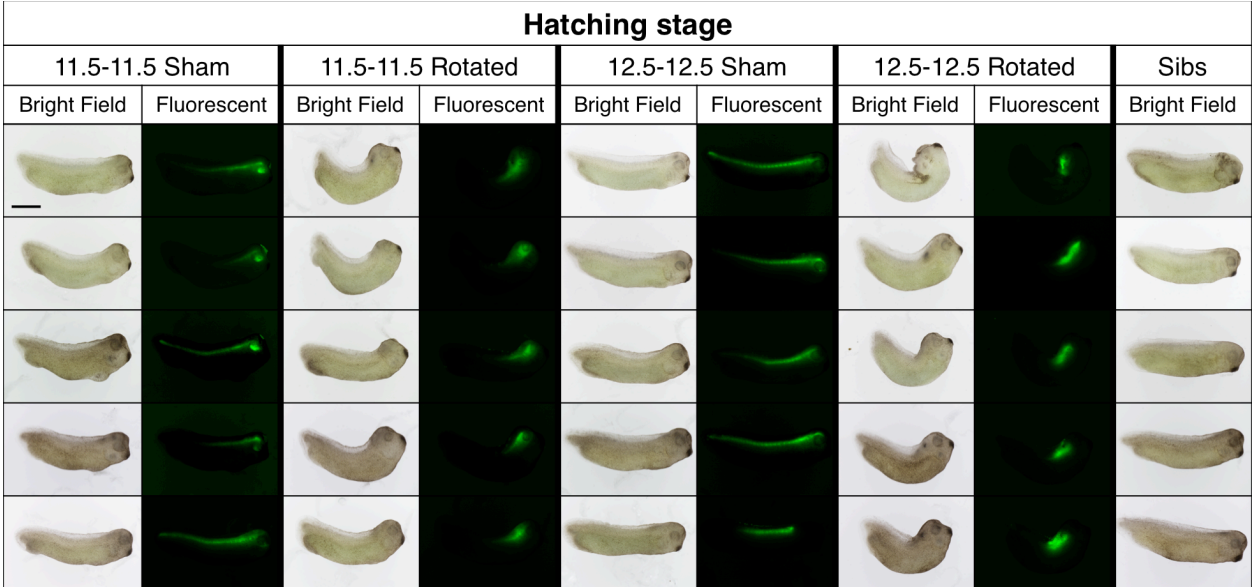
Figure 21: Hatching stage embryos used for RNA-Sequencing analysis. Fluorescent image indicates location or transplant incorporation. Lateral view of all embryos with dorsal facing up; anterior is to the right. Scale bar represents 1mm.

Differential Gene Expression

No genes were significantly differentially expressed between 11.5-11.5 Sham and

Rotated embryos at either stage 18 or stage 30. Between 12.5-12.5 Sham and Rotated embryos,

there was one differentially expressed gene at stage 18 (Table 1) and 10 differentially expressed

genes at stage 30 (Table 2). Tables 1 and 2 display normalized transcript counts for each

condition in transcripts per kilobase million (TPM) (Wagner, Kin, & Lynch, 2012), as well as the

fold change of gene expression and the adjusted p value between the conditions. A positive fold

change indicates that the gene is upregulated in 12.5-12.5 Rotated embryos compared to Sham,

while a negative number indicates downregulation.

Table 1: Differentially expressed genes between 12.5-12.5 Sham and 12.5-12.5 Rotated embryos at stage 18. TPM = transcripts per kilobase million. A positive fold change (highlighted in blue) indicates that the gene is upregulated in 12.5-12.5 Rotated embryos compared to Sham, while a negative number (highlighted in red) indicates downregulation Log_2 Fold Change is based on absolute read counts.

Gene	TPM		Log_2 Fold Change	padj
	12.5-12.5 Sham	12.5-12.5 Rotated		
EGFL6	0	4	1.236	1.30E-3

Table 2: Differentially expressed genes between 12.5-12.5 Sham and 12.5-12.5 Rotated embryos at stage 30. TPM = transcripts per kilobase million. A positive fold change (highlighted in blue) indicates that the gene is upregulated in 12.5-12.5 Rotated embryos compared to Sham, while a negative number (highlighted in red) indicates downregulation Log_2 Fold Change is based on absolute read counts.

Gene	TPM		Log_2 Fold Change	padj
	12.5-12.5 Sham	12.5-12.5 Rotated		
HARBI1	0	1	1.055	5.57E-05
NRL	4	2	-0.782	1.45E-3
PLCD1	1	3	0.685	2.75E-3
ATOH7	13	5	-0.811	0.0117
GSX1	9	4	-0.722	0.0274
PTF1A	12	5	-0.782	0.0274
PROM1	7	3	-0.677	0.0282
PTBP3	8	11	0.401	0.0282
TRPV2	2	6	0.822	0.0282
PRDM13	4	2	-0.663	0.0338

Expression of Regional Marker Genes

The four regional marker genes assayed by *in situ* hybridization in previous experiments did not show differential expression between treatment groups (Tables 3-6). Tables 3-6 display normalized transcript counts for each condition in transcripts per kilobase million (TPM), as well as the fold change of gene expression and the adjusted p value between the conditions.

Table 3: Expression of regional marker genes between 11.5-11.5 Sham and 11.5-11.5 Rotated embryos at stage 18. TPM = transcripts per kilobase million. Log_2 Fold Change is based on absolute read counts.

Gene	TPM		Log_2 Fold Change	padj
	11.5-11.5 Sham	11.5-11.5 Rotated		
XCG-1	0	0	0.0119	1.00
Otx2	100	92	-0.0509	1.00
En-2	19	23	0.172	1.00
Krox20	15	19	0.243	1.00

Table 4: Expression of regional marker genes between 11.5-11.5 Sham and 11.5-11.5 Rotated embryos at stage 30. TPM = transcripts per kilobase million. Log_2 Fold Change is based on absolute read counts.

Gene	TPM		Log_2 Fold Change	padj
	11.5-11.5 Sham	11.5-11.5 Rotated		
XCG-1	0	0	-0.00332	1.00
Otx2	55	54	-0.0181	1.00
En-2	7	12	0.425	1.00
Krox20	2	2	0.0206	1.00

Table 5: Expression of regional marker genes between 12.5-12.5 Sham and 12.5-12.5 Rotated embryos at stage 18. TPM = transcripts per kilobase million. Log_2 Fold Change is based on absolute read counts.

Gene	TPM		Log_2 Fold Change	padj
	12.5-12.5 Sham	12.5-12.5 Rotated		
XCG-1	0	0	-0.0127	1.00
Otx2	91	92	-0.0966	1.00
En-2	21	23	-0.0258	1.00
Krox20	13	11	-0.109	1.00

Table 6: Expression of regional marker genes between 12.5-12.5 Sham and 12.5-12.5 Rotated embryos at stage 30. TPM = transcripts per kilobase million. Log$_2$ Fold Change is based on absolute read counts.

Gene	TPM		Log$_2$ Fold Change	padj
	12.5-12.5 Sham	12.5-12.5 Rotated		
XCG-1	0	0	0.330	0.574
Otx2	66	48	-0.400	0.0910
En-2	7	9	0.239	0.881
Krox20	1	1	0.125	0.969

Sibling Gene Expression

The expression of Sham transplants was compared with that of unperturbed sibling embryos to address possible differences between these groups of embryos. High levels of differential gene expression were found between Sham and sibling embryos (Table 7).

Table 7: Numbers of significantly differentially expressed genes between sibling and Sham or Sham and Rotated embryos. 7A shows these comparisons for stage 18, while 7B shows them for stage 30.

Comparison (Stage 18)	Number of DE Genes
11.5-11.5 Sib vs. Sham	632
11.5-11.5 Sham vs. Rotated	0
12.5-12.5 Sib vs. Sham	791
A. 12.5-12.5 Sham vs. Rotated	1

Comparison (Stage 30)	Number of DE Genes
11.5-11.5 Sib vs. Sham	152
11.5-11.5 Sham vs. Rotated	0
12.5-12.5 Sib vs. Sham	7
B. 12.5-12.5 Sham vs. Rotated	10

Discussion

Homochronic Transplants

The ability of embryos to respond to perturbations from their environment is an essential feature of normal development. Although the development of the anterior-posterior neural axis in *Xenopus laevis* has been previously studied, the extent of plasticity of this axis in response to perturbations is not known. In this study, we investigated the response of *Xenopus laevis*

embryos to a physical perturbation of their developing neural axis using transplantation experiments. In addition to classic embryological techniques, we used molecular markers to assess the patterning of the anterior-posterior axis after transplantation. Our results identify a window of plasticity during which *Xenopus laevis* embryos are able to recover from a 180-degree anterior-posterior rotation of their neural axis. Expression of regional marker genes indicates that embryos have the ability to successfully re-pattern their AP axis following inversion at the mid-gastrula stage, but lose this ability by the late-gastrula stage. This inability to re-pattern the neural axis at the late-gastrula stage is not due simply to a loss of healing ability as embryos progress through gastrulation; late-gastrula stage embryos with non-rotated Sham transplants were able to develop phenotypically normally and show correct expression of regional marker genes.

This result is consistent with previous studies of neural axis inversion. Roach (1945) performed axis rotation experiments on pre-neurula and neurula stage *Ambystoma punctatum* embryos and found that the rotated tissue retained the identity of its previous position in the embryo. Since the window of plasticity identified in our study is closing around the late-gastrula stage, it is reasonable to expect that the window would remain closed as development proceeds to the pre-neurula and neurula stage. Waddington and Yao (1950) performed anterior-posterior rotations on the Organizer in embryos just beginning gastrulation, and saw complete recovery for most of the embryos in their study. These two studies define a wide range of time during which the neural axis becomes committed in its patterning: from early-gastrula stage to pre-neurula stage. Our study narrows this window to a period between the mid- and late-gastrula stage.

Our finding of a closing window of plasticity is also consistent with Sive's finding that embryos are only able to be re-patterned with retinoic acid during a small window of time in

development (1990), suggesting a labile period for re-patterning ability. Saha and Grainger's (1992) experiments on *Xenopus laevis* demonstrated that marker genes have initially large regions of expression along the AP neural axis earlier in gastrulation, which become more restricted as gastrulation proceeds. The initial broad possible areas of expression of regional marker genes at the mid-gastrula stage demonstrate that specific cells have not yet committed to a precise regional fate, and suggest that they have some plasticity to respond to changes in their signaling environment.

After transplantation, embryos were grown to either the late-neurula or hatching stage before being fixed and analyzed. We hypothesized that recovery from transplantation may not be complete by the late-neurula stage, and we would see a gradual increase in the extent of compensation as development progressed. This hypothesis is somewhat supported by our results. For both morphology and expression of regional marker genes, very similar patterns are evident at stage 18 and 30. At both stages, 12.5-12.5 Rotated embryos have the highest proportion of abnormally developing embryos and the lowest levels of correct gene expression. 12.5-12.5 Rotated embryos only have significantly higher levels of ectopic gene expression at stage 30 and not at stage 18, suggesting that 12.5-12.5 Rotated embryos show progressive de-regulation and inability to compensate for the initial perturbation. In contrast, 11.5-11.5 Rotated embryos have an increasing proportion of normally developing embryos from stage 18 to stage 30, suggesting that these embryos are able to compensate progressively better as development proceeds. However, since no direct statistical comparisons were made between stage 18 and stage 30, this interpretation must be considered with caution.

Heterochronic Transplants

The second part of this study aimed to determine the relative contribution of the underlying mesoderm and overlying ectoderm to the loss of neural axis plasticity over time using heterochronic transplants. Previous studies have investigated the age of transplanted tissue as a factor in determining the competence or plasticity of that tissue. Albers (1987) transplanted neural ectoderm between donors and hosts of different stages of *Ambystoma mexicanum* and assayed the amount of neural tissue induced in each case. He found the age of the transplant from the donor to be the deciding factor in the amount of neural tissue produced; older transplants led to the induction of less neural tissue. Based on this idea that the competence of the overlying ectoderm is essential to neural induction, we hypothesized that 11.5-12.5 transplants would recover from transplantation and rotation better than 12.5-11.5 transplants. However, our results do not support this hypothesis.

The clearest difference identified in our results is between 12.5-11.5 Sham embryos and the other three experimental treatments. 12.5-11.5 Sham embryos were the most likely to exhibit normal morphology at both the late-neurula and hatching stage, and also had the lowest ectopic expression of regional marker genes at these stages. Contrary to Albers's findings, this result suggests that the age of the mesodermal signaling environment is an important factor in determining the ability of neural ectoderm to develop properly. Presumptive neural ectoderm from a stage 11.5 embryo is unable to regulate when transplanted into a stage 12.5 host. In contrast, homochronic transplant experiments demonstrate that stage 11.5 presumptive neural ectoderm regulates properly when transplanted into a stage 11.5 host. Even if stage 11.5 neural ectoderm is competent to respond to patterning signals, stage 12.5 mesoderm could have stopped producing the proper signals. Another hypothesis is that stage 11.5 ectoderm transplanted into a

stage 12.5 host is unable to properly undergo the process of convergent extension. This process begins at stage 10.5 as the lateral edges of the neural epithelium converge inward and the anterior and posterior ends extend outward, eventually leading to the formation of the neural folds and neural tube (Keller & Danilchik, 1988). The primary force of convergent extension comes from the movement of mesodermal cells (Keller & Danilchik, 1988). Stage 11.5 ectoderm may not be able to develop properly when transplanted into a stage 12.5 host because it is effectively missing out on two hours of convergent extension movements.

11.5-12.5 Rotated and 12.5-11.5 Rotated embryos did not differ from each other in morphology, New On scores, or Old Off scores. Both of them failed to fully regulate following rotation of their AP neural axis. This result suggests that in the case of neural axis inversion, both the ectoderm and the mesoderm are crucial in determining the plasticity of the AP neural axis. Despite not agreeing with our initial hypothesis, this result makes sense. Studies performed on neural competence such as those of Albers (1987), focused simply on the induction of neural ectoderm and not its patterning. The development of a correct neural axis pattern following AP axis rotation is a complex process that requires both neural ectoderm and underlying mesoderm to retain a degree of plasticity.

RNA-Sequencing

Differential Gene Expression

Results from RNA-Sequencing of homochronic transplants support the conclusions of morphology and marker gene analysis. Embryos with neural axis transplantation performed at stage 11.5 show no differential gene expression between Sham and Rotated transplants, indicating that Rotated transplants are able to successfully regulate gene expression following a

rotation of their AP axis at stage 11.5. In contrast, embryos showed differential gene expression between Sham and Rotated transplants performed at stage 12.5.

The one differentially expressed gene between 12.5-12.5 Sham and 12.5-12.5 Rotated embryos at stage 18 is *EGFL6* (epidermal growth factor-like domain, multiple 6). Although the expression of *EGFL6* has not yet been published in *Xenopus*, it has been found to be involved in zebrafish notochord development (Wang, Wang, Yuan, Chai, & Liu, 2015). *EGFL6* has also been identified in two recent studies of amputation. It was found to be upregulated following limb amputation in *Ambystoma mexicanum* (Campbell et al., 2011) and in the regenerating tail blastema of the lizard *Podarcis muralis* (Vitulo, Dalla Valle, Skobo, Valle, & Alibardi, 2016). Campbell et al. (2011) suggest that *EGFL6* may play a role in the epidermal growth that is a necessary part of the wound healing process. *EGFL6* was upregulated in 12.5-12.5 Rotated embryos compared to Sham embryos, which could mean it is being recruited more heavily for wound healing in 12.5-12.5 Rotated embryos.

The ten differentially expressed genes between 12.5-12.5 Sham and Rotated embryos at stage 30 are primarily genes involved in neural development, especially development of the retina. *NRL* (neural retinal leucine zipper) is required for the development of rod photoreceptor cells (Mears et al., 2001) and can reprogram retinal precursors to have a rod fate (McIlvain & Knox, 2007). *Atoh7* (atonal bHLH transcription factor 7) is required for the development of retinal ganglion cells and has been found to be regulated by FGF signaling (Willardsen et al., 2009). *Prom1* (prominin 1) is involved in the development of the disk arrays of rod and cone photoreceptors (Han, Anderson, & Papermaster, 2012; Kleinman & Ambati, 2008). These three genes are all downregulated in 12.5-12.5 Rotated embryos compared to 12.5-12.5 Sham embryos. This is consistent with the expression pattern of our regional marker gene *Otx2*, which

is expressed in the eye and forebrain. *Otx2* was the only one of the four regional marker genes to approach significant differential expression between 12.5-12.5 Sham and 12.5-12.5 Rotated embryos at stage 30 (padj = 0.09). As with the other eye-related genes, *Otx2* was downregulated in 12.5-12.5 Rotated embryos compared to 12.5-12.5 Sham embryos. *Otx2* is essential for the development of the anterior nervous system (Pannese et al., 1995) and controls the fate of retinal progenitor cells (Nishida et al., 2003; Viczian, Vignali, Zuber, Barsacchi, & Harris, 2003). The downregulation of these genes related to eye-development suggests that 12.5-12.5 Rotated embryos have abnormal development of their eyes by stage 30.

Several of the other candidate genes are also involved in neural development and patterning. *Gsx1* is expressed in the forebrain and hindbrain in *Xenopus tropicalis* starting around the neural plate stage (Illes, Winterbottom, & Isaacs, 2009). *Gsx1* is directly involved in early neural patterning, by regulating the expression of BMP and the Iroquois family homeobox genes, both key regulators of neural patterning (Winterbottom, Ramsbottom, & Isaacs, 2011). *Ptf1a* (pancreas specific transcription factor, 1a) is involved in pancreatic development, but also is a downstream target of Wnt/beta-catenin signaling (McLin, Rankin, & Zorn, 2007) and is a powerful inducer of GABAergic neurons, the primary inhibitory neuronal subtype (Dullin et al., 2007). Another candidate gene, *Prdm13* (PR domain zinc finger protein 13) is a downstream target of *Ptf1a*, and also serves to promote a GABAergic fate in neurons (Hanotel et al., 2014). *Prdm13* is also a histone methyltransferase (Hanotel et al., 2014), which points to a possible role for epigenetic changes in regulating neural development after axial rotation. *Gsx1, Ptf1a,* and *Prdm13* are all downregulated in 12.5-12.5 Rotated embryos compared to 12.5-12.5 Sham embryos.

Ptbp3 (polypyrimidine tract binding protein 3) is expressed in specific subdomains of the brain and spinal cord, suggesting that it may play a role in neural patterning (Noiret, Audic, & Hardy, 2012). It is involved in many aspects of RNA processing (Romanelli, Diani, & Lievens, 2013), and helps with the localization of *Vg1* RNA to the vegetal pole of *Xenopus* oocytes, a key step in the early patterning of the embryo (Lewis, Gagnon, & Mowry, 2008). *TrpV2* is a non-specific cation channel that is activated by heat, and is expressed in sensory neurons (Lee, Shim, & Oh, 2005). Although it has not been extensively studied in *Xenopus laevis,* it has been found to begin expression at the neurula stage in *Xenopus tropicalis* (Silina, Nikishin, & Kremnyov, 2015) and to be involved in regulating axon outgrowth in mice (Shibasaki, Murayama, Ono, Ishizaki, & Tominaga, 2010). *Ptbp3* and *TrpV2* are both upregulated in 12.5-12.5 Rotated embryos compared to 12.5-12.5 Sham embryos.

The last two differentially expressed genes between 12.5-12.5 Sham and 12.5-12.5 Rotated embryos at stage 30 do not have as clear connections to neural development. The most highly differentially expressed gene is *Harbi1* (harbinger transposase derived 1), which has an unknown function and has not been studied in *Xenopus* (Kapitonov & Jurka, 2004). Despite its name, *Harbi1* does not have transposase activity, but is predicted to have nuclease activity (Kapitonov & Jurka, 2004). *Harbi1* is very conserved among bony vertebrates, suggesting that it may be important for their development (Kapitonov & Jurka, 2004). In a recent study of spinal cord injury in zebrafish, Hui et al. (2014) found that *Harbi1* was upregulated both in uninjured fish and in fish seven days post-injury. *PLCD1* (phospholipase C delta 1) has also not been studied in *Xenopus*, but functions as a tumor suppressor in humans (Xiang et al., 2010). *Harbi1* and *PLCD1* are both upregulated in 12.5-12.5 Rotated embryos compared to 12.5-12.5 Sham embryos.

Expression of Regional Marker Genes

The four regional marker genes analyzed in this study (*XCG-1, Otx2, En-2, and Krox20*) were detected by RNA-Sequencing, but were not found to have any differential expression among treatment groups. This is seemingly contradictory with our *in situ* hybridization analysis of marker gene expression, which found significant differences in the expression of these genes among the treatment groups. However, the groups found to be significantly different from each other in our *in situ* analysis were not the same groups we compared for the RNA-Seq analysis. For the *in situ* analysis, we chose to run an ANOVA to determine if any global differences were present before proceeding with post-hoc analysis. With the RNA-Seq experiment, we wanted to minimize the number of comparisons made and proceeded directly to pairwise comparisons of the most biologically relevant pairs (Sham vs. Rotated).

RNA-Sequencing also loses the important spatial dimension of gene expression. Our analysis of *in situ* hybridization of these marker genes was based on their location; New On scores were assigned for gene expression in the correct, endogenous location, while Old Off scores were assigned for gene expression in incorrect, ectopic locations. Based on this scoring system, we found not only decreased correct expression in 12.5-12.5 Rotated embryos, but also increased ectopic expression. Considering the RNA-Seq results alongside the *in situ* hybridization results supports the conclusion that the overall level of these regional marker genes remains relatively unchanged following reversal of the AP axis, but their spatial distribution along the axis is perturbed.

The regional marker gene *XCG-1* was found to have high levels of expression in *in situ* hybridization assays, but had a TPM of zero for all categories in the RNA-Seq experiment. Although this result is strange, it is consistent with the massive RNA-Seq analysis recently

performed on *Xenopus laevis* by Session et al. (2016), which also found that *XCG-1* had a TPM of zero across all developmental stages.

Sibling Gene Expression

It is worth noting that the overall number of DE genes between transplant conditions was relatively small. In comparison, many more genes were differentially expressed between transplant embryos and sibling embryos. For example, 1 gene was differentially expressed between 12.5-12.5 Sham and Rotated embryos at stage 18, while 791 genes were differentially expressed between 12.5-12.5 Sham and sibling embryos at stage 18 (Table 7A). This vast difference suggests that the main genetic response following transplantation is due to an injury response and the stress of incorporating new donor tissue rather than the re-patterning of the AP axis.

Another possible reason for the large difference in gene expression between siblings and transplant embryos is a time delay of development following injury. Transplant embryos were fixed based on the age of unperturbed siblings, and there was a noticeable lag in the development of transplant embryos compared to siblings. When sibling embryos reached stage 18, Sham embryos were closer to stage 16 or 17 (Fig. 20). Thus, the large number of DE genes between Sham and sibling embryos could just be the normal amount of differential gene expression between a stage 16 and stage 18 embryo. By stage 30, Sham transplants seem to have caught up to sibling embryos (Fig. 21). This could explain the decreased number of DE genes between sibling and Sham embryos at stage 30 (Table 7B). Unfortunately, there is not a current *Xenopus* RNA-Seq data set that includes samples from stages 16, 17, and 18 that we can use to test this hypothesis. Future RNA-Seq experiments on a wider range of Sibling embryo stages would allow us to tease apart the reason for this large amount of differential gene expression.

Limitations

Several limitations of this study must be considered. The first and most major of these concerns the co-localization of transplanted neural ectoderm with the regional marker genes assayed with *in situ* hybridization. Sometimes the region of transplanted tissue, as marked by an anti-fluorescein antibody, did not overlap with the endogenous expression location of the gene of interest. For example, *XCG-1*, which is expressed in the far anterior cement gland, did not co-localize with transplanted ectoderm that incorporated in the posterior spinal cord. As we were interested in the contribution of the *transplanted ectoderm* to re-patterning after transplantation and/or rotation, we specifically wanted to look at gene expression that was *co-localized* with transplanted tissue; this specification is included in our definition of New On. Embryos that did not have transplant incorporation in the location of endogenous gene expression were instead scored in the category Host On. This indicates the ability of the host tissue to correctly bring up regional marker gene expression following transplantation, but not the transplant. While this is interesting, it does not answer the same question as New On expression and thus Host On scores were not presented in our results.

Some transplant treatments were more likely to be affected by the New On/Host On distinction than others. For example, 12.5-12.5 Rotated embryos often fail to undergo proper convergent extension following transplantation and thus tended to have very small regions of transplant incorporation that often did not co-localize with anterior neural markers. In contrast, 11.5-11.5 Sham embryos tended to have proper extension and transplant incorporation down the entire length of the AP axis. However, all embryos were still able to be scored for Old Off; even if the transplanted tissue was not in the location where endogenous expression would be, it could still be analyzed for any ectopic gene expression. In addition, The RNA-Seq experiment helps

address these concerns by investigating global gene expression instead of just the expression of four regional marker genes.

Another limitation to consider is the ability of Sham embryos to recover after transplantation. The *in situ* hybridization experiments indicate that they do not always have perfect expression of regional marker genes, and the RNA-Seq experiments demonstrate a large number of differentially expressed genes between Sham and sibling embryos. This shows that even in the case where neural ectoderm is not rotated, embryos still have some difficulty recovering from the massive perturbation of having their neural ectoderm removed and replaced with another piece of ectoderm. It is likely that the pieces of ectoderm removed from the donor and host embryo were from slightly different positions. Although embryo shape and pigmentation was used successfully to reliably identify the presumptive neural ectoderm, it is impossible to guarantee that the exact same square of ectoderm down to the sub-millimeter level was removed from the donor and host embryo. However, Sham embryos still regulated more completely than Rotated embryos, and serve as a control for the effects of injury and wound healing.

Future Directions

Differentially expressed genes from the RNA-Seq dataset are candidate genes for future investigation. On the bioinformatics side, we plan to further analyze these genes using pathway analysis or genome ontology. We also plan to validate our RNA-Seq results by characterizing the expression of the candidate genes with *in situ* hybridization. The first step is to successfully clone these genes and create working RNA probes, a process that is currently underway. Once effective probes have been made, we will validate the RNA-Seq results by comparing *in situ*

gene expression in the experimental conditions between which we originally found differential

expression of the gene. After this analysis of expression, we can perform functional

characterization of these genes by either knocking them down or overexpressing them and

observing the effects on neural axis plasticity. The most promising candidate genes are those that

have been previously implicated in neural development and patterning, although all of them will

be interesting to investigate further.

A lingering question from the RNA-Seq data set is why there are so many differentially

expressed genes between sibling embryos and Sham transplant embryos. One possibility for this

difference is that slightly different regions of neural ectoderm were transplanted from donor to

host, requiring even Sham transplants to undergo some extra amount of regulation to correct for

these slight changes in position. Another possibility is that these gene expression differences

relate to the developmental lag witnessed even in Sham embryos; when sibling embryos had

reached stage 18 and were ready to be fixed, Sham embryos appeared to be only around stage 16

or 17. These options are not mutually exclusive, as the requirement for slight re-patterning could

have contributed to the lag in development. In order to address this question, a new RNA-Seq

dataset is currently being collected using an "autotopic" transplant system. In this method, a

square of presumptive neural ectoderm is excised from a mid- or late-gastrula stage embryo and

then replaced into exactly the same position it was before. In this experimental setup, the embryo

still has to recover from a significant surgery and incorporate an explant of neural ectoderm.

However, the explant is patterned exactly the same as the underlying mesoderm. The downside

of this method is that the incorporation of the transplanted piece of ectoderm cannot be

visualized, as the transplantation is not occurring between a labeled donor embryo and an

unlabeled host embryo. However, this system does help control for the effects that slight

mispositions in transplant location may have on the Sham healing process, and could serve as a better control to investigate the effects of neural axis rotation. Preliminary results indicate that autotopic transplants performed at either stage 11.5 or 12.5 and fixed at stage 18 or stage 30 have normal morphology and little to no developmental lag compared to sibling embryos (Fig. 22).

A.   B.
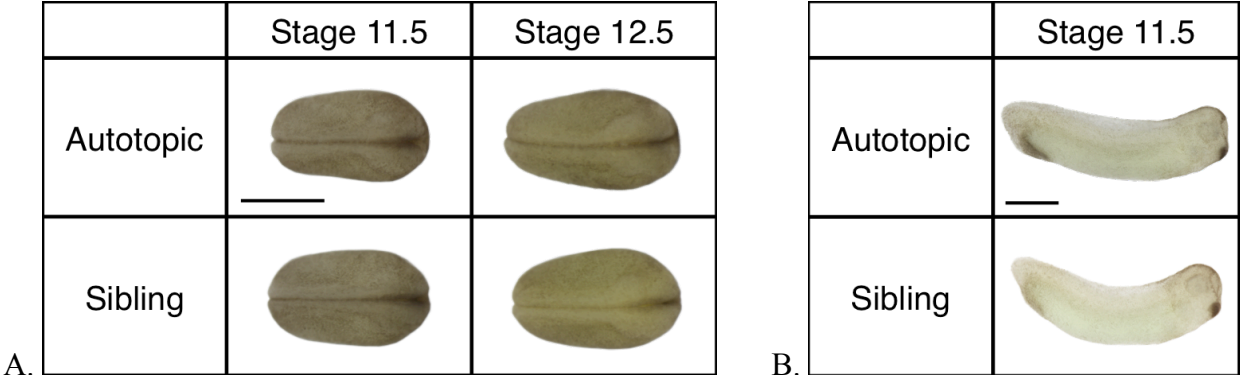
Figure 22: Development of embryos following autotopic transplantation. 22A shows embryos grown to stage 18; dorsal view with anterior to the right. 22B shows embryos grown to stage 30; lateral view with anterior to the right. Scale bar represents 1mm in both 22A and 22B.

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