

Nitric oxide as a putative retinal axon pathfinding and target recognition cue in *Xenopus laevis*

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Nitric oxide (NO) is an atypical neurotransmitter synthesized by the enzyme nitric oxide synthase (NOS) during many stages of the *Xenopus laevis* life cycle. This research investigates whether the gas NO is involved in axon guidance, the neurodevelopmental process in which axons travel through the brain to their appropriate target locations to form functional neural circuitry. Through immunocytochemistry and direct labeling of the NO gas with a fluorescent dye, we have found that NOS expression corresponds spatiotemporally with the beginning of retinal axon innervation of the optic tectum in *X. laevis*. Our function-blocking studies in which NO is chemically inhibited suggest that NO may be necessary for correct pathfinding and targeting, evidenced by qualitative widening of the optic tract and aberrant target innervation.

Abbreviations: NO – nitric oxide, NOS – nitric oxide synthase, RGC – retinal ganglion cell, OT – optic tectum, L-NAME – L-nitro arginine methyl ester

Keywords: nitric oxide synthase, retinotectal system, axon target recognition

Introduction

Axon guidance refers to how axons pathfind, or travel, from one location in the nervous system to another. Proper and appropriate axon guidance in a developing organism is necessary for the formation of neural circuits and, consequently, the transfer of information throughout the nervous system. The retinotectal system, which involves the axons that travel from the retina to the optic tectum, is a model researchers use to investigate the mechanisms underlying axon guidance. *Xenopus laevis*, the African clawed frog, is an appropriate model organism to use in the study of retinotectal axon guidance because it produces hundreds of embryos per mating round, progresses rapidly through different developmental stages, and is sufficiently large to allow for manipulation, microsurgery, dissection and axon tract labeling (Chien and Harris, 1994). Furthermore, retinal axon morphology and progression is well-documented and understood in *X. laevis*, which allows inhibition and over-expression studies to be performed as

the resulting phenotypes can be compared to a known standard of normal axon guidance (Chien and Harris, 1994).

The retinal ganglion cell (RGC) axons that ultimately form the circuitry of the retinotectal system during development first exit the eye, then enter the brain at the location of the ventral diencephalon, cross at the optic chiasm to enter the contralateral optic tract, and travel along the tract, turning caudally towards the optic tectum (OT). The OT is the location in *X. laevis* where visual input from the retina is sent (Harris and Holt, 1990). These axons travel at a relatively uniform speed and only slow down once they near the optic tectum, the target location where arborization, or unbundling of the axon tract and innervation of the target region, occurs (reviewed in Harris and Holt, 1990). When the embryos are housed at 22°C, it takes the axons 13 hours to travel the approximately half millimeter distance from the chiasm to the optic tectum (Harris et al., 1985).

A number of molecules, termed axon-guidance cues, function to help the axons migrate along the appropriate, stereotyped path (Harris and Holt, 1990). A subset of these axon guidance cues serve to assist with target recognition; these cues instruct the RGC axons to recognize the target region, reduce migration speed, and stop advancing, and arborize once the optic tectum is reached (Harris and Holt, 1990).

Although many axon guidance cues have been elucidated, not much is known about the cues that guide *X. laevis* RGC axons from the retina to the optic tectum. One currently being investigated by researchers is nitric oxide (NO), a gaseous free radical classified as a neurotransmitter. Unlike typical neurotransmitters, however, NO is not stored in vesicles (Bruning and Mayer, 1996). In humans, NO has a variety of different functions, from the regulation of blood flow to acting as the response to action potentials in the thalamus (reviewed in Vincent, 2010). Although axon growth cones in snail neurons lengthen in response to NO donors (Van Wagenen and Rehder, 1999), the role of NO in axon guidance has yet to be firmly elucidated.

In culture, NO has been shown to act as a repulsive cue on dorsal root ganglion cell axon growth cones that are plated on a laminin substrate, but as an attractive cue on growth cones that are plated on an L1 substrate (Tojima et al., 2009). Given that numerous different extracellular matrix and substrate proteins are present *in vivo*, it is unknown whether NO will act as a repulsive cue or an attractive cue, or possibly both depending on concentration or presence of varying extracellular matrix and cell adhesion molecules, in the actual *X. laevis* embryo. Researchers have deduced that NO is necessary for the refinement of established axonal projections in the frog, but this is a later stage of neural development preceded by axon guidance (Wu et al., 2001; Cline and Constantine-Paton, 1990).

Current research on the expression of nitric oxide synthase (NOS), the enzyme responsible for the production of NO, has found NOS positive cells in the optic tectum of the chick, fish, and frog (reviewed in Giraldi-Guimardes et al., 2007). In teleost fish, NOS immunoreactivity was found in the retinal

ganglion cells and could be traced along these axons to the optic tectum (Gaikwad, 2009). The precise timing of the expression of NOS in *X. laevis*, however, is disputed in the literature. Some researchers have suggested that expression does not begin until premetamorphic stage 46 (Lopez and Gonzalez, 2002), while others propose that NOS protein is first made at stage 35-36, when axons turn caudally to reorient themselves towards the tectum (Cogen and Cohen-Cory, 2000). Consequently, the timing of onset of expression of NOS needs to be defined and clarified. As stated previously, the majority of the data on the role of NO with axons is in regards to the refinement of established axonal connections, not on the formation of axonal connections during development. In the presence of NO donors, branches, which are projections off of the primary axon tract, are added to established axon tracts, but in the presence of NOS inhibitors, there is an increase in the length of individual axon branches, but no net increase in the number of branches (Cogen and Cohen-Cory, 2000). In late stages (55-60, when the majority of the organisms are froglets) of *X. laevis* RGC axon explants in culture, the application of an NO donor led to reduced motility or complete growth cone collapse in the majority of the growth cones analyzed (Renteria and Constantine-Paton, 1999). However, this cannot be definitively extrapolated to the growth cones of RGCs as they travel from the retina to the optic tectum, since these RGCs are from earlier stage embryos and refinement is not yet occurring, because the axons have not yet reached their final destination in the optic tectum.

In the chick visual system, expression of NOS is temporally linked to axon innervation of the optic tectum, so it is possible that nitric oxide plays a role in keeping axons on the path to the optic tectum (Williams et al., 1994). Furthermore, NO has been found to slow neurite outgrowth of *Helisoma* B5 neurons (Trimm and Rehder, 2004); this slowing-down phenomenon is necessary once the axons begin to near the target region so that the axons innervate the appropriate area and a functioning neural circuit is formed. Based on these functions of NO in other systems, it is possible that NO may act as

an axon guidance cue in RGC axon guidance in *X. laevis*.

This research is looking to address the hypothesis that NO plays a role in axon pathfinding along the optic tract to the optic tectum. We hypothesized that if NO does indeed have a function in this portion of retinotectal circuit formation, NOS and NO gas are likely expressed in the optic tectum and that inhibiting NO will cause axon guidance defects. The embryos examined were stage 35-36 embryos, when the axons reach the middle of the optic tract and turn caudally towards the tectum, stage 37-38 embryos, when the first axons reach the optic tectum, and stage 39-40 embryos, when the majority of axons have reached the optic tectum and have begun the arborization process (Chien and Harris, 1994).

It is important to study axon guidance as aberrant axon guidance is implicated in a number of diseases and neurodevelopmental pathologies. For example, the over-expression in adults of semaphorins, netrins and slits, established axon guidance molecules that normally govern the development of neuronal circuits, could possibly lead to the destruction of functioning neural circuits in Amyotrophic Lateral Sclerosis (Schmidt et al., 2009). Given that NO plays many important roles in the adult human nervous system, perhaps understanding its role in the development of the nervous system during axon guidance will allow for a better of understanding of its myriad functions, both in normal and pathological states.

Material and Methods

All reagents are from Sigma-Aldrich, Saint Louis, MO, unless otherwise noted.

X. laevis embryo fixation

X. laevis embryos were obtained from in-house natural matings with Human Chorionic Gonadotropin (HCG)-injected adult females. All of the protocols detailed in this manuscript were IACUC approved carried out according to the Haverford College IACUC guidelines. The embryos were then grown at either 16°C or 22°C to regulate development, as the embryos mature faster at higher temperatures. Embryos of the desired developmental stage (staging according to observational criteria established

by Nieuwkoop and Faber, 1956) were fixed in MEMFA (8:1:1 ratio of ddH₂O, 37% formaldehyde, and 10X MEM salts) or Dent's (20% Dimethyl Sulfoxide (DMSO) and 80% MeOH) fixatives for anti-NOS and anti-neurofilament (anti-NF) immunocytochemistry, respectively. Different antibodies require different fixation conditions for optimal results, and this must be worked out for each antibody in question (Sive et al., 2000). Consequently, the embryos were fixed in different solutions depending on the antibody staining procedure that was to follow dissection, as NF works optimally in Dent's fixative, while anti-NOS works optimally in MEMFA fixative. Fixed embryos were stored in 100% EtOH (MEMFA fixative) or 100% EtOH (Dent's fixative) at -20°C until use.

X. laevis embryo brain dissection

Petri dishes were coated with a solution of 1% agar prepared in 1X Marc's Modified Ringer's solution to create a dissecting dish. The agarose mixture was allowed to solidify to provide a supportive surface on which to pin down and dissect the embryos. Using no. 5 watchmaker's forceps, halved micro-pins were inserted through the embryo into the agar in two locations: directly behind the eye and in the middle of the tail. This kept the embryo in place during the dissection process. Using an insect pin, the skin covering the eye was gently peeled off and removed using the watchmaker's forceps. Next, the insect pin was used to separate the brain tissue from the rest of the embryo. The brain tissue was then used immediately for immunocytochemical analyses or stored in 100% EtOH at -20°C for later use.

Anti-NOS immunocytochemistry

10-15 MEMFA-fixed brain tissue samples each dissected from the appropriate stage (35, 37, 40) whole embryo first underwent a series of washes in TBS buffer and 0.3%-Triton-X-TBS to rehydrate and permeabilize the tissue. Next, the brain tissue samples were incubated in 20% Fetal Calf Serum (FCS) diluted in 1X TBS to prevent non-specific binding of the antibody. After blocking, the brain tissue samples were incubated with a

1:500 dilution of anti-NOS1 primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) prepared in 20% FCS. A set of control embryos was incubated in the blocking solution without the primary antibody to ensure that the staining seen after the substrate reaction was not merely non-specific background binding. After TBS buffer washes, the samples were incubated in a 1:100 dilution of goat anti-rabbit alkaline phosphatase tagged secondary antibody (Sigma-Aldrich, Saint Louis, MO). Finally, a substrate reaction was performed to localize the presence of the NOS antibody-tagged protein by adding the alkaline phosphatase BCIP/NBT substrate (SIGMAFAST™ BCIP®/NBT, Sigma-Aldrich, Saint Louis, MO) to the brain tissue samples. The brain tissue samples were allowed to incubate in the substrate solution for approximately 5 minutes or until the blue localization color appeared. Samples were then visualized and imaged using a Nikon DXM microscope and NIS elements imaging software.

DAF-FM (4-Amino-5-methylamino-2',7'-difluorescein) diacetate labeling of NO gas

The diaminofluorescein DAF-FM is a reagent that fluorescently labels and localizes NO (Kojima et al., 1999). The DAF-FM diacetate effectively labels the NO gas because it is a dye that emits an increased amount of fluorescence after it reacts with an intermediate of the spontaneous reaction of NO to NO₂ (Sheng et al., 2005; Kojima et al., 1999). DAF-FM diacetate (Molecular Probes, Invitrogen, Carlsbad, CA) was diluted to a 1mM concentration in DMSO. The experimental embryos (approximately 10-15 embryos per stage) were bathed in a 5μM solution of DAF-FM prepared in 0.1X MMR for 30 minutes, while control embryos (approximately 5-10 embryos per stage) were bathed in 0.1X MMR for 30 minutes so that the autofluorescence of the embryos could be examined. After the bath application, the embryos were fixed in MEMFA fixative as detailed above. For image acquisition, the FITC/GFP filter on a Nikon DXM camera-equipped microscope and NIS elements imaging software were used. After images of the whole embryo were acquired, the brain tissue was dissected and images of isolated

brain tissue were subsequently taken. An outlining procedure was performed in Adobe Photoshop to show that the embryos that did not fluoresce, and were subsequently not seen in the captured images, were actually present under the microscope. The control images were overexposed using the auto contrast feature, which picks up miniscule amounts of fluorescence, allowing a rough outline of the embryo to be seen. This overexposed image was then traced using the paintbrush feature and the tracing was copied to a non-overexposed copy of the original image, showing where the embryo would have been located. Tracing of the experimental samples was done for consistency, though the overexposure was not performed when the embryo could be visualized clearly without doing so.

L-NAME (L-nitro arginine methyl ester) NO inhibition studies

L-NAME (Sigma-Aldrich, Saint Louis, MO) is a chemical inhibitor of NO production. Its structure is markedly similar to that of L-arginine, the starting material for NO synthesis. Consequently, L-NAME exerts its effects by competing with L-arginine, which is normally converted to L-citrulline and NO by NOS (Knowles et al., 1989). For the inhibition studies, approximately 20-30 stage 30-31 embryos were incubated in a solution of either 5μM L-NAME prepared in 0.1X MMR or pure 0.1X MMR, the latter serving as a control. The embryos were allowed to develop until they reached stage 39-40, when the majority of the axons have reached their target of the optic tectum. Retinal axons were then labeled using the anti-NF labeling method described below.

Anti-NF immunocytochemistry for L-NAME inhibition studies

Anti-NF labeling is a means to label axons that is significantly less technically demanding than other procedures, such as horseradish peroxidase or fluorescent intercalation labeling. Consequently, anti-NF labeling is used to increase sample size or when timing prohibits HRP labeling, which must be done on live tissue. It is important to note, however, that anti-NF labeling does not only

label retinal axons, especially near the optic tectum. Other axons also express the cytoskeletal neurofilament protein to which the antibody binds; the tightly bound retinal axons of the optic tract allow for clear labeling in that area, but once the retinal axons begin to reach the optic tectum they begin to spread out and labeling becomes less specific for retinal axons. Consequently, HRP anterograde labeling is still the preferred method for retinal axon tract labeling. Recently, however, the protocol was optimized for labeling of retinal axons by incubating the tissue in a lower concentration of primary antibody (1:1000 vs. 1:100 dilution) for a longer time (for 2 days as opposed to overnight) and including more washes (6 or 7 washes as opposed to 4 washes); this allows more efficient labeling of the retinal axons near the optic tectum and reduces non-specific background in that region. Dissected Dent's fixed brain tissue samples underwent a procedure similar to that described above for the anti-NOS immunocytochemistry labeled samples. The primary antibody used was a 1:1000 dilution of anti-NF antibody (Invitrogen, Carlsbad, CA) and the secondary antibody used was a 1:100 dilution of goat anti-rabbit antibody (Sigma-Aldrich, Saint Louis, MO). Finally, the brain tissue was imaged using a NikonDXM camera-equipped microscope and NIS Elements imaging software. On Adobe Photoshop, the width of the RGC tract was measured using the ruler tool. The optic tract width measurements for the treated and non-treated samples were averaged, and the standard deviation was calculated using Microsoft Excel. A non-paired, two-tailed Student's t-test was also performed using Microsoft Excel.

Results

NOS Immunocytochemistry

In stage 37 *X. laevis* NOS antibody-incubated brain tissue, there is blue colored staining, which indicates NOS expression in the area in and around the optic tectum (Figure 1C, I). The tectum is identified based on morphological landmarks given in Chien and Harris (1994); the small raised bump in the midbrain region is where the tectum is located in the mid 30s to mid 40s developmental stages. In

the control samples, which were not treated with the primary antibody, there is no localization of NOS staining in or around the OT (Figure 1D, J). In brain tissue dissected from stage 35 and stage 40 embryos; however, the experimental brain tissue incubated in the primary antibody does not appear markedly different from the negative control brain tissue (Figure 1A, B, E, F, G, H, K, L). In some of the samples, both in the experimental and control tissue, there is an accumulation of pigment cells in the tectal area, which must be differentiated from true staining. There is no localization of blue staining in the OT as in the stage 37 brain tissue, which leads to the conclusion that the NOS synthase protein is not expressed in stage 35 and stage 40 brain tissue. In sum, NOS is expressed near the tectum in stage 37, but not stage 35 or 40 *X. laevis* embryos.

NO Expression Studies (DAF-FM Labeling)

In 67% (n=6) of stage 35 whole embryos incubated in the DAF-FM diacetate solution, green fluorescence, which signifies presence of the NO gas, is observed, but the fluorescence does not extend fully into the dorsal region where the brain is located (Figure 2A). In 33% (n=3) of stage 35 embryos, however, there is little to no expression where the eye is located. For the stage 37 embryos treated with DAF-FM diacetate, all of the embryos (n=6) examined show NO expression in the area around the eye, similar to the stage 35 embryos. In stage 37 embryos, the NO expression does not extend completely into the dorsal region where the brain is located (Figure 2B). In stage 40 embryos, all samples (n=7) have NO expression around the eye region (Figure 2D). In these samples there appears to be a complete absence of fluorescence in the regions where the brain is located, whereas in the stage 35 and 37 samples, the fluorescence in that region is dim but not non-existent (Figure 2C). Overall, NO gas is expressed near developing neural structures in all embryonic stages examined (Figure 2D).

FIGURE 1

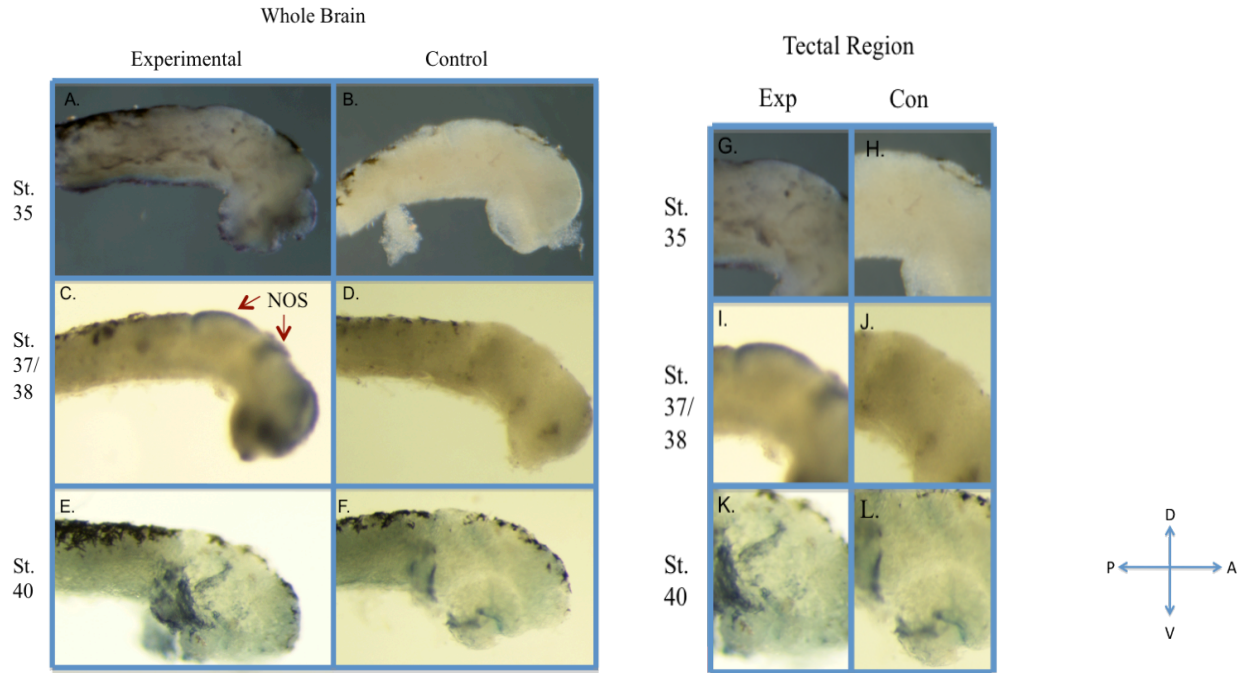


Figure 1: NOS is expressed in the optic tectal region of stage 37 brain tissue. Experimental denotes primary antibody added, control denotes no primary antibody. In stage 35 experimental (A: whole brain; G: close up of tectal region) there is no defined blue (NOS) staining. The control brain tissue (B: whole embryo; H: tectal region) is clean and has low background. In stage 37 experimental, there is a defined blue region (NOS) in and around the optic tectum (C: whole embryo, see red arrows; I: tectal region). Stage 37 control brain tissue (D: whole embryo; J: tectal region) is clean and has low background. Staining is observed in stage 40 experimental (E: whole brain; K: tectal close up), but is likely only background and not NOS expression, as it is similar to background staining in control (F: whole brain; L: tectal region). All images 60X.

In brain tissue dissected from stage 35 embryos, the fluorescence is only slightly more intense in the treated samples (n=13) than in the control samples (n=7) (Figure 3A,B). Consequently, the level of NO in stage 35 brain tissue is likely relatively low, if present at all. In the stage 37 brain tissue, however, the intensity of fluorescence is markedly increased in the treated samples (n=9) compared to the control samples (n=4). In some of the stage 37 samples the NO expression is concentrated throughout the brain tissue (n=3); in other samples (n=6), however, the expression is more localized in the region just above the notochord and posterior to the retinal axon tract (Figure 3C). In the brain tissue isolated from stage 40 embryos, the samples incubated with the DAF-FM diacetate (n=8) do not differ in fluorescence intensity from the control samples (n=4). This leads to the conclusion that NO is not present at appreciable

levels in the brain tissue of stage 40 embryos (Figure 3E,F). In sum, NO gas is expressed in brain tissue from stage 37 embryos, but not in brain tissue from stage 35 or 40 embryos (Figure 2G).

L-NAME Inhibition Studies

Following incubation in either the NO inhibitory solution or the control solution, anti-NF immunocytochemistry was performed on the brain tissue samples, leading to successful labeling of the optic tract. This labeling of the optic tract is necessary to observe phenotypic differences between the drug-treated and control samples. The two main defects observed in the brain tissue from L-NAME treated embryos (n=7) are premature widening of the optic tract and targeting errors. Compared to the embryos which were incubated in 0.1X MMR (n=5), the

FIGURE 2

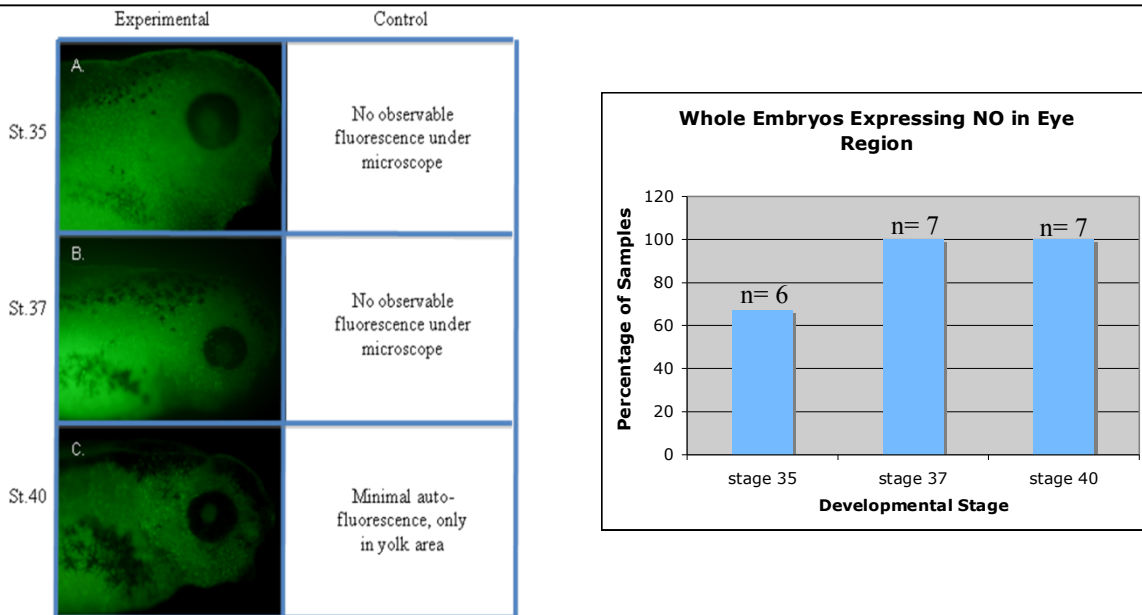


Figure 2: Nitric oxide gas is expressed in stage 35-40 whole embryos. Experimental denotes DAF-FM diacetate dye added, control denotes no dye added. In the majority (67%) of the stage 35 whole embryos, NO is expressed in the region surrounding the eye and where the brain is located, but with a lesser intensity in the brain region; A is a representative image. All stage 37 whole embryos examined have expression of NO surrounding the eye and into the brain region, but lesser intensity in brain region; B is a representative image. In stage 40 embryos, all have expression in the region surrounding the eye, but the staining does not extend into the region where the brain is located; C is a representative image. D shows percentage of DAF-FM treated embryos that express NO in eye region. All images 80X.

FIGURE 3

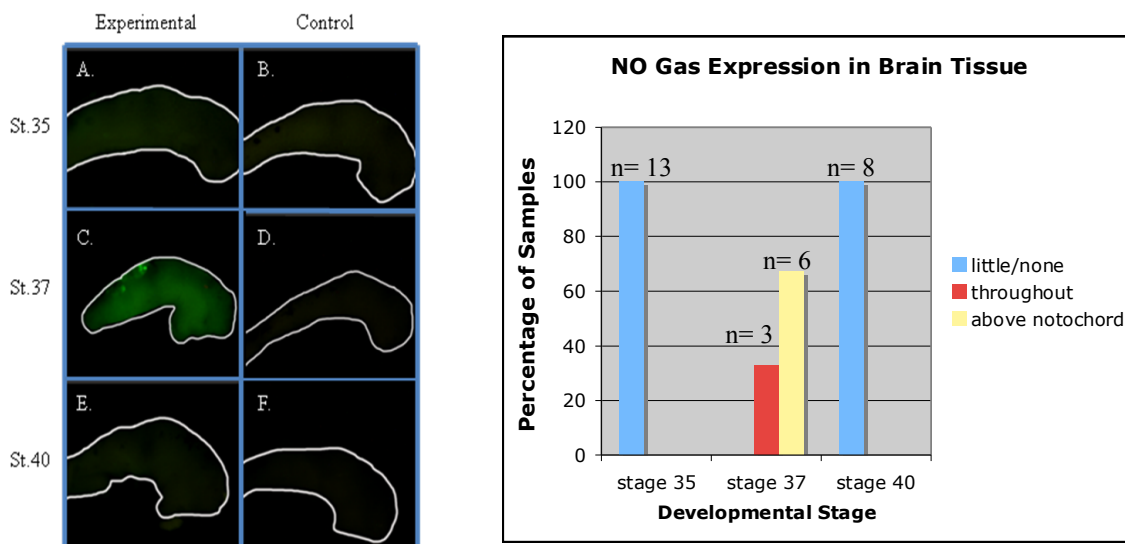


Figure 3: NO gas is expressed in stage 37 brain tissue: Experimental denotes DAF-FM diacetate dye added, control denotes no dye added. In stage 35 and stage 40 there is not a significant observable difference between the experimental (A, E) and control embryos (B, F). In stage 37, the experimental brain tissue (C) glows green (NO+) while the control (D) does not. The stage 37 expression is concentrated in the region above the notochord posterior to where the retinal axon tract would be located. G is a graphic representation of the pattern of NO gas expression in the developmental stages examined. All Images 115X.

optic tract of the L-NAME embryos appears qualitatively wider in at least 2 out of the 7 samples (Figure 4A). From this data, we conclude that NO appears to be required for normal RGC axon guidance along the optic tract. A paired, two-tailed student's t-test, however, gave a non-significant p value of 0.077 (Figure 4B).

Discussion

The procedure to determine whether a particular molecule is an axon guidance cue is two-fold; data must be obtained about both the expression and function of the molecule in question. In order for a molecule to be classified as an axon guidance cue, it must have a

spatiotemporal pattern of expression that agrees with the developmental time period and the location in which the axons are extending. In the case of retinal axon pathfinding from the ventral diencephalon to the optic tectum, this means that the putative cue must be present in at least some of the developmental time period between stage 32 and stage 40. Furthermore, the cue must be expressed in or near the region where the retinal axons are extending. Given that NOS and NO are likely expressed in stage 37 of *X. laevis* development, when the first retinal axons have begun to reach the optic tectum, NO satisfies this first spatiotemporal expression requirement for classification as an axon guidance molecule.

FIGURE 4

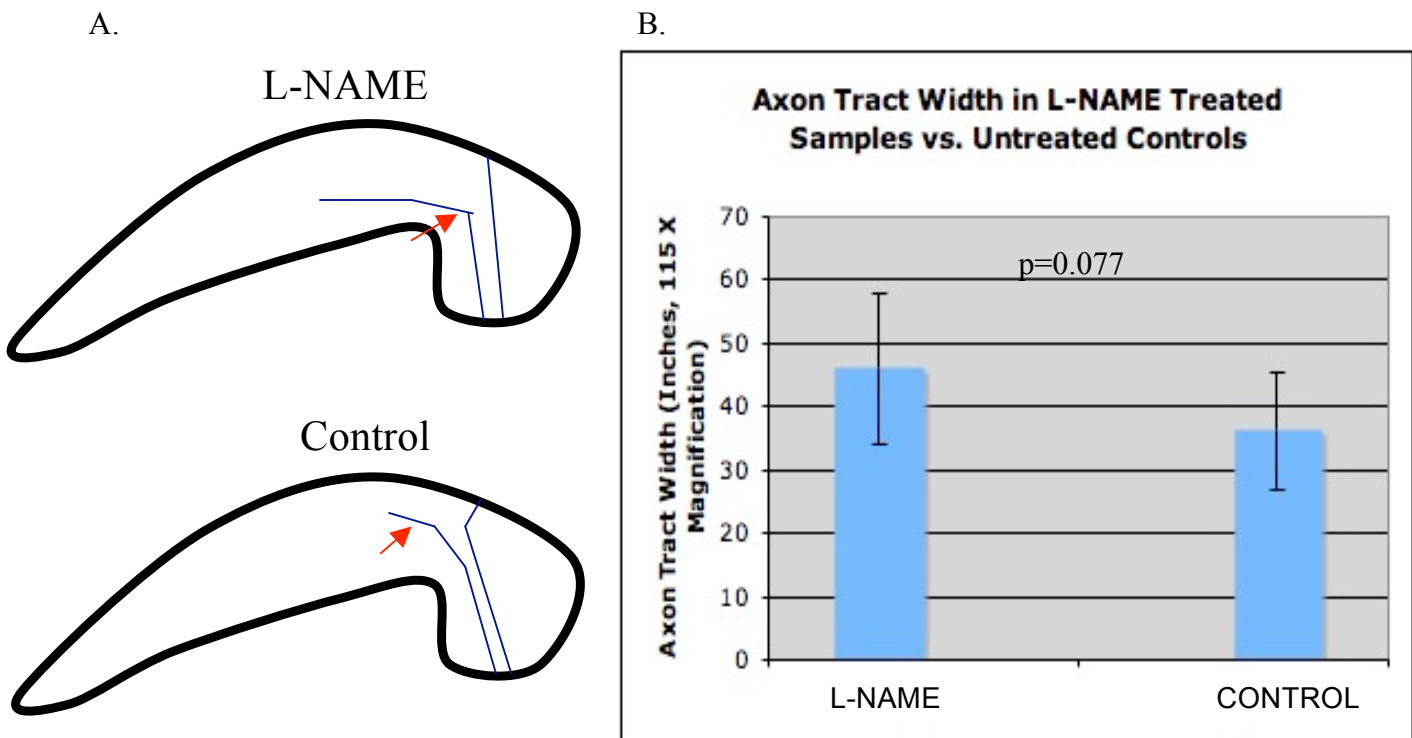


Figure 4: Schematic and quantification of observed axon extension phenotype upon treatment with L-NAME. A. In the L-NAME treated brain tissue, there is earlier arborization of the optic tract than observed in the controls, denoted by the red arrows. Arborization signifies targeting, so the axons are targeting more ventrally in the L-NAME treated sample than in the control. B. There is also qualitative widening of the optic tract as it pathfinds over the neuroepithelium in the treated samples compared to the control samples, though the change in width is not statistically significant (p value of 0.077, two-tailed paired Student's t-test).

NOS Expression Studies

We found that NOS, the enzyme responsible for NO production, is expressed in the optic tectal region in brain tissue from stage 37 *Xenopus* embryos, but not in the brain tissue from stage 35 or 40 embryos. This clarifies conflicting data from previous NOS expression studies, which reported expression beginning in such varying developmental time periods as stage 35-36 to stage 46 (Cogen and Cohen-Cory, 2000; Lopez and Gonzalez, 2002). The spatiotemporal expression in stage 37 suggests that NOS is likely involved more so in the inception of axon targeting, which begins in stage 37-38 of development, rather than in the earlier pathfinding processes that occur in stage 35-36 embryos.

NO Expression

An important step in our process to determine that NO indeed satisfied the appropriate spatiotemporal expression characteristic of a guidance cue was to assay for the presence of the NO gas. Although our immunocytochemistry data showed that the enzyme responsible for the production of NO was present, it did not provide us with direct information about the presence of the NO gas itself. With many enzymes, it is possible that the product may be transported away from the site of synthesis. This is especially an issue when the products are gases, which are often diffusible in nature, although they could be held in place to some degree by the presence of an extracellular matrix. From the DAF-FM diacetate staining data, we can conclude that although the NO gas does diffuse away from the site of synthesis (the tectal region) to some degree, it remains in the brain tissue of stage 37 *X. laevis* embryos.

In some of the stage 37 samples the NO gas is located throughout the entirety of the brain tissue (Figure 3C), however, in the majority of others the gas is more localized to the ventral region and posterior to the path of axon travel (image not shown). These different expression patterns suggest different possible functions for NO in retinal axon guidance. If it is indeed expressed throughout the brain tissue, perhaps it functions to keep the axons traveling throughout the brain until they reach the optic tectum, where another guidance cue signals

them to stop traveling and innervate the optic tectum. When varying concentrations of an NO donor were applied to the growth cones of snail axons from B5 neurons in culture, the response varied depending on the amount of donor applied. With low concentration of the donor, neurite extension begins to slow, with an intermediate concentration, neurite outgrowth stops, and with a high concentration, growth cones collapse. Given the expression of NO in stage 37 embryos, when the axons must recognize and innervate the tectum, it is possible that NO is acting as a “slow-down and stop” signal as it does in snail axons (Trimm and Rehder, 2004). Although the NO gas is not only expressed in the optic tectum, perhaps it is the contact with the NO that causes the axons to stop extending, which subsequently signals they should innervate the region they are approaching. The optic tectum may be the source of another guidance cue, but for the axons to fully respond to this other cue at the optic tectum, they might need to be first sufficiently slowed by NO.

Netrin-1 and Engrailed are molecules expressed in the optic tectum during axon pathfinding or targeting (reviewed in Mann et al., 2004; Wizenmann et al., 2009); these are hypothetical guidance cues to which NO may allow the axons to respond. Another possibility for the function of NO that corresponds with the ventral and posterior staining pattern is that NO is acting as a repulsive guidance cue. The gas is preventing the axons from migrating into the area more ventrally than is appropriate, keeping them on the appropriate track to the tectum. In fact, NO was found in some instances to act as a repulsive axon guidance cue in dorsal root ganglion cell axons in chick (Tojima et al., 2009). It is important to recognize, however, that we cannot make the direct conclusion from this data that NO will serve the exact same purpose in *X. laevis* retinal axon guidance.

NO Inhibition Studies

The functional studies allow us to make more definitive hypotheses as to the specific role of NOS and NO in retinal axon guidance in *Xenopus* by showing us the morphological defects when NO is inhibited. Although the widening of the optic tract in the

L-NAME treated samples is not statistically significant, on examination of some of the samples, the optic tract does in fact appear considerably wider. These observations suggest that further quantitative analyses be performed on a larger number of brain tissue samples that is more consistently labeled to see if there is indeed a significant widening of the optic tract. The qualitative widening of the optic tract observed in the L-NAME treated embryos could be due in part to the presence of NO throughout the brain tissue keeping the axons tightly bundled together.

In fact, the neural cell adhesion molecule (NCAM), which is known to play a role in neurite outgrowth, has been found to exert its effects through interaction with the NO-cGMP signaling cascade. NCAM is suggested to function in neurite outgrowth via the production of cGMP to activate protein kinase G; NCAM increases the concentration of cGMP by activating soluble guanylyl cyclase through NO (Ditlevsen et al., 2007). Perhaps the presence of NO throughout the brain tissue is necessary for NCAM to function properly and keep the tract tightly bundled. The targeting errors, evidenced by the premature arborization and innervation of the retinal axons in the L-NAME treated samples, suggest that NO is required in some manner for the axons to reach and appropriately recognize the optic tectum as the location to stop advancing. If NO is not interacting appropriately with NCAM, this could possibly lead to the failure of the axons to advance to the tectum. This lack of advancement could then aberrantly signal to the axons that they had reached their target and should arborize, which would account for the inappropriate innervation/arborization phenotype observed. NO-cGMP staining has been found to be characteristic of migrating gut cells in the grasshopper (Knipp and Bicker, 2009); it is possible that the retinal axons also require NO-cGMP signaling in order to advance successfully. Given that during the process of axon guidance, RGCs essentially must migrate from the retina to the OT, there is at least a tenuous functional similarity between *X. laevis* axon guidance and grasshopper cell migration.

Another functional possibility comes from combining the ventral/posterior NO expression data with the targeting errors from

the functional studies. In the L-NAME treated brain tissue, the axons appear to arborize, or branch out, in a more posterior/ventral location than appropriate. This widening appears to extend into the region of maximum NO staining observed in the majority of the brain tissue samples. Perhaps the NO gas is acting as a repulsive cue by preventing the axons from extending into the region where the NO gas is most heavily expressed; it is only once the axons reach the tectum where other cues are located that arborization and innervation of the tissue occurs. This is a similar function to that proposed for sonic hedgehog, a protein axon guidance cue that is roughly expressed in the region where we observe maximum NO expression (Gordon et al., 2010). Contrary to the pattern of expression of sonic hedgehog, which is not expressed outside of this ventral region posterior to the optic tract, NO is expressed, albeit more weakly. Given that many axon guidance cues are morphogens, which act based on concentration gradients, the NO expression pattern could mean that NO is functioning in axon guidance in a morphogenic fashion (Zou and Lyuksyutova, 2007). Other axon guidance cues expressed in the diencephalon that may also interact with NO include Slits, whose expression in the ventral diencephalon borders the optic tract (like that of NO), and Sema3A, which is expressed in the forebrain (Atkinson-Leadbeater et al., 2010, reviewed in Erskine and Herrera, 2007). Slits and Sema3a serve a similar repulsive function to that hypothesized for NO by keeping the axons traveling along the optic tract, which suggests the possibility of an interaction between NO and these cues.

The data from these experiments shows that NOS is not significantly expressed in the brain tissue until stage 37 and, to these researchers' knowledge, is the first study to stain for the presence of the NO gas in *X. laevis* brain tissue during the time period in which retinal axon guidance occurs. The gas labeling experiments are extremely important, as they show that the active molecule, NO gas, has an appropriate spatiotemporal expression for axon guidance. If only NOS expression had been examined, definitive conclusions about the role of NO could not have been made. The functional studies, although only preliminary, when

combined with the expression data, suggest an important functional role for NO in keeping the axons traveling appropriately along the optic tract and preventing premature arborization and targeting.

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