Inhibition of Retinoic Acid Receptors Results in Defasciculation of the Trigeminal Nerve in Xenopus laevis

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INHIBITION OF RETINOIC ACID RECEPTORS RESULTS IN
DEFASCICULATION OF THE TRIGEMINAL NERVE IN XENOPUS
LAEVIS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science
at Virginia Commonwealth University

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Receptor expression of candidate regulators of trigeminal development

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<tbody>
<tr>
<td>3A10</td>
<td>Neurofilament-associated protein antibody</td>
</tr>
<tr>
<td>6G7</td>
<td>B-tubulin antibody</td>
</tr>
<tr>
<td>ALCAM</td>
<td>Activated leukocyte cell adhesion molecule</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>AP-2</td>
<td>Activating protein 2 (Neural crest marker)</td>
</tr>
<tr>
<td>B-CAM</td>
<td>Basal cell adhesion molecule</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolyl phosphate</td>
</tr>
<tr>
<td>BMB</td>
<td>Boehringer Mannheim Blocking Agent</td>
</tr>
<tr>
<td>BMS-453</td>
<td>Retinoic acid receptor inhibitor</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>DIG</td>
<td>Digoxigenin</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>MAB</td>
<td>Maleic acid buffer</td>
</tr>
<tr>
<td>MBS</td>
<td>Modified Barth’s saline</td>
</tr>
<tr>
<td>NaBH₄</td>
<td>Sodium borohydride</td>
</tr>
<tr>
<td>Nav2</td>
<td>Neuron navigator 2</td>
</tr>
<tr>
<td>NBT</td>
<td>Nitro blue tetrazolium</td>
</tr>
<tr>
<td>NEDD9</td>
<td>Enhancer of filamentation 1</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NT-3</td>
<td>Neurotrophin-3</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PBT</td>
<td>Phosphate buffer saline with Tween</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoic acid</td>
</tr>
<tr>
<td>RALDH (1-3)</td>
<td>Retinaldehyde dehydrogenase (1, 2 and 3)</td>
</tr>
<tr>
<td>RAR</td>
<td>Retinoic acid receptor</td>
</tr>
<tr>
<td>RARE</td>
<td>Retinoic acid response element</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
</tr>
<tr>
<td>Sema4B</td>
<td>Semaphorin 4B</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------</td>
</tr>
<tr>
<td>SSC</td>
<td>Saline-sodium citrate</td>
</tr>
<tr>
<td>SHH</td>
<td>Sonic hedgehog</td>
</tr>
<tr>
<td>SRCR</td>
<td>Scavenger receptor cysteine-rich domain</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>Trk (A-C)</td>
<td>Tropomyosin-related kinase (A, B and C)</td>
</tr>
<tr>
<td>VAD</td>
<td>Vitamin A deficient</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>µm</td>
<td>Micron</td>
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Abstract

INHIBITION OF RETINOIC ACID RECEPTORS RESULTS IN DEFASCICULATION OF THE TRIGEMINAL NERVE IN XENOPUS LAEVIS

By Jeremy A Thompson, B.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University

Major Director: Amanda J Dickinson, Ph.D.
Assistant Professor, Department of Biology

The anatomy of the cranial peripheral nervous system has been studied for over a century, yet surprisingly little is known about how the nerves are guided to their targets. The study of the development of these nerves has important implications for our understanding of
craniofacial anomalies and possible treatments for both injury and genetic disorders of nerve development such as Goldenhar-Gorlin syndrome. We have discovered that retinoic acid (RA) may play a role in the development of the trigeminal nerve. Inhibition of retinoic acid receptors (RAR) results in trigeminal nerves that become unbundled or defasciculated in the eye region. To further understand how RA is affecting trigeminal development we searched for genes downregulated in response to RAR inhibition by the inhibitor BMS-453 and have identified neurotrophin-3 (NT-3), activated leukocyte cell adhesion molecule (ALCAM) and Semaphorin 4B (Sema4B). We have analyzed the expression patterns of Sema4B and NT-3 by in situ hybridization and have found NT-3 expression in the eye and Sema4B in the embryonic target of the trigeminal nerve, lens of the eye and in the pharyngeal arches. ALCAM has been analyzed via qRT-PCR and its transcription is downregulated just prior to the observed defasciculation phenotype. The pattern of expression of these genes combined with known expression of NT-3 receptors allows us to suggest a model whereby RA signaling regulates Sema4B, ALCAM and NT-3, which support the survival, guidance and fasciculation of the trigeminal nerve. This work has the potential to better understanding of the complex nature of cranial nervous system development.
Introduction

The trigeminal nervous system is critical for mediating our interaction with the environment. This system allows us to taste and chew food, provides the sense of touch to the majority of our facial epidermis and allows us to communicate with others. The sensory fibers of the trigeminal system carry information for touch as well as visceral sensation and position sense and taste; while the motor fibers innervate muscles of mastication. Understanding how the development of the trigeminal nerve is regulated is important for our understanding of congenital abnormalities of the trigeminal nerve such as Goldenhar-Gorlin syndrome and could aid in the prevention of such maladies (Nappi, Alfonsi et al. 1983; Das, Ray et al. 2008; Schroder, Lassig et al. 2013).

Anatomy of the trigeminal nervous system

The cranial nerves are numbered I-XII, of which the trigeminal nerve is the fifth (O'Rahilly 1988; Kandel, Jessell et al. 2000) (see Supp Table 1 and 2 for review). The pattern of trigeminal innervation is distinct and conserved in all vertebrate model organisms studied. The trigeminal nerve consists of three main branches: the ophthalmic, maxillary and mandibular (Streeter 1908; Hamburger 1961; Davies, Kitson et al. 1982; Martin 1996). The ophthalmic branch is sensory and extends anteriorly to arborize in the tissue around the eye, frontal, and nasociliary areas (Streeter 1908; Davies, Kitson et al. 1982; Kuratani and Tanaka 1990; Martin 1996). The maxillary and mandibular branches extend ventrally to innervate the jaws (Streeter
1908; Martin 1996; Schlosser and Northcutt 2000). The maxillary nerve separates from the combined maxillomandibular nerve at the maxillary process and turns anteriorly to provide sensation to the upper jaw, palate, teeth and the upper oral cavity (Streeter 1908; Davies, Kitson et al. 1982; Martin 1996). The mandibular nerve projects ventrally to the future lower jaw following Meckel’s cartilage and is a ‘mixed’ nerve containing sensory and motor fibers for muscles of mastication (Hamburger 1961; Martin 1996; Kandel, Jessell et al. 2000).

**Development of the trigeminal nervous system**

The development of the sensory components of the trigeminal nerve requires the interaction of two cell types: neural crest and ectodermally derived placodes (Blentic, Chambers et al. 2011). The placodes are transient columnar epithelia that arise from ectoderm lateral to the neural tube and are believed to be specified by factors emanating from the dorsal portion of the neural tube as it closes (Streit 2004; McCabe and Bronner-Fraser 2009). Neural crest cells are a population of cells that arise from dorsal ectoderm adjacent to the neural tube and are defined by two characteristics: they are highly migratory and are self-renewing pluripotent stem cells (Donoghue, Graham et al. 2008; Muller and O'Rahilly 2011). The neural crest of the head region, the cranial neural crest, gives rise to sensory neurons, glial cells, and nonneural cell types (Muller and O'Rahilly 2011). Whereas the placodal cells of the future trigeminal ganglia give rise exclusively to sensory neurons (McCabe and Bronner-Fraser 2009). Placodal cells delaminate and undergo a dorso-medial migration to the site of the future ganglia during which they become post-mitotic expressing neural markers (Schlosser and Northcutt 2000; Schwarz,
Vieira et al. 2008; Blentic, Chambers et al. 2011). The cranial neural crest component of the trigeminal system is derived from ectoderm adjacent to a specific segment of the hindbrain; rhombomere 2 (Begbie and Graham 2001; Schwarz, Vieira et al. 2008; Blentic, Chambers et al. 2011). Neural crest migrate ventrally and laterally to the site of the future ganglia where they act to guide the migrating placodal neuroblasts (Hamburger 1961; Begbie and Graham 2001; Schwarz, Vieira et al. 2008).

There is much variation amongst species in the relative contribution of each cell type to the future trigeminal ganglia (Hamburger 1961; Schlosser and Northcutt 2000; Begbie and Graham 2001; Streit 2004; McCabe and Bronner-Fraser 2009; McCabe, Sechrist et al. 2009). Overall, it appears that placodally derived neurons contribute to the vast majority of the trigeminal nervous system while the neural crest make minor contributions to the overall neuronal population but are immensely instrumental in the organization of the ganglia and neural projections both centrally and peripherally (Hamburger 1961; Barlow 2002; Schwarz, Vieira et al. 2008). The development of the motor fibers of the mandibular branch is less complex since these are not derived from the placodes and neural crest; rather they come from tissue within the hindbrain (Guthrie 2007).

The development of the trigeminal ganglia and nerves has been studied in mouse, chick, and in Xenopus as well as human. In Xenopus the fused profoundal and trigeminal ganglia form similarly to mammals and birds (Nieuwkoop 1967; Davies, Kitson et al. 1982; Davies 1988; Kuratani and Tanaka 1990; Muller and O'Rahilly 2011). They first appear at stage 21(22hpf)
just after the neural tube has closed and begin sending out the first neurites between stages 22 and 24 (24hpf) just as the neural crest is migrating prior to differentiation of the orofacial structures. In *Xenopus* the mandibular nerve is the first cranial nerve to appear (stage 22/23, 24hpf) and projects ventrally toward the first pharyngeal arch and cement gland reaching it by stage 28 (32hpf) (Davies, Kitson et al. 1982). The cement gland is a transient structure that allows the embryo to attach to surfaces. Sensory trigeminal innervation of the cement gland commences a reflex which stops swimming upon contact with a surface (Boothby and Roberts 1992). The maxillary nerve also arises at stage 22/23 (24hpf) and follows the contour of the optic stalk which directs it anterior and dorsal to the mandibular nerve (Davies, Kitson et al. 1982).

The development of the trigeminal nervous system is a very complex process involving numerous cells of different origin coming together to form a common structure. Much of the anatomy of the trigeminal nervous system is conserved evolutionarily, yet there is still variation making exact comparisons between development in one organism with that in another difficult. In addition, a multitude of genes and signaling pathways regulate the development of the trigeminal nervous system.

**The role of retinoic acid in trigeminal nervous system development**

The vitamin A derivative retinoic acid (RA) is a small molecule that has diverse functions in developing embryos and the adult organism (reviewed in (Duester 2008; Kam, Deng et al. 2012)). Vitamin A is converted to retinoic acid in a series of oxidation reactions involving enzymes including retinaldehyde dehydrogenases (RALDHs 1-3) (reviewed in (Duester 2008)).
RA is known to exert its effects on gene transcription through two classes of nuclear receptors, retinoic acid receptors (RARα,β, and γ) and retinoid X receptors (RXRα,β, and γ)(reviewed in (McGrane 2007)). Once in the nucleus, RA binds to its receptors and can regulate the transcription of genes which have retinoic acid response elements (RAREs) in their promoter region (Mark, Ghyselinck et al. 2009). In *Xenopus* RALDH2 is predominantly expressed along with RARγ in a complementary and overlapping expression pattern in the orofacial region of *Xenopus laevis* (Kennedy and Dickinson 2012).

RA has a role in cranial nervous system development such as its role in delineating the boundaries between segments of the hindbrain, rhombomeres 2 and 3 as well as 4 and 5 by controlling temporal as well as spatial Hox gene expression (Kam, Deng et al. 2012). RA along with sonic hedgehog (SHH) in the spinal cord are responsible for establishing a ventral motoneuron fate and regulating neurogenesis by transcriptional control of genes (Duester 2008; Lee, Lee et al. 2009).

In addition to RA’s role in the differentiation and patterning of the nervous system, it has also been implicated in axonal outgrowth and guidance (Carter, Farrar et al. 2010; Kam, Deng et al. 2012). Carter et al. (2010) have shown that RA has the capability to induce axon turning toward the source of RA by signaling through RXRα. This signaling of RA has been shown multiple times and in different species (Liao, Ho et al. 2004; Calderon and Kim 2007; Carter, Farrar et al. 2010). Other works have shown that RA influences the transcription of genes known to guide axons such as NEDD9, Neurogenin-2, Nav2 and Semaphorin 6B (Correa, Sasahara et al.
RA has been well characterized for its role in cranial nervous system development, especially the posterior cranial nerves (Clagett-Dame, McNeill et al. 2006; Guthrie 2007; Duester 2008). While there is evidence that RA is required for trigeminal development less is known about the mechanism of RA signaling in development of the trigeminal nervous system (Clagett-Dame, McNeill et al. 2006). Neurite outgrowth from the trigeminal ganglia is also disrupted in vitamin A-deficient (VAD) rats (Clagett-Dame, McNeill et al. 2006). In rescue experiments of VAD rats by supplementation with RA at levels low enough to rescue hindbrain patterning defects was observed without rescuing aberrant cranial nerve projections. This data indicate a role for RA in the guidance of these neurites (Clagett-Dame, McNeill et al. 2006). In addition, RARs and RXRs are expressed in the trigeminal ganglia and other RA pathway components are expressed in the motor nuclei of the trigeminal system further supporting their role in trigeminal development (Ruberte, Friederich et al. 1993; Georgiades, Wood et al. 1998). RA may also indirectly affect the development of the trigeminal nerve by influencing the development of target tissues (Kennedy and Dickinson 2012).

In the model system *Xenopus laevis*, RA signaling components are expressed in the correct place and at the correct time to influence trigeminal nerve development. Given the previously described roles of RA in nervous system development it is possible that RA could have a role in the differentiation, patterning, outgrowth or guidance of the trigeminal nervous
system in *Xenopus*. Taken together, previous studies have shown that RA can influence the development of the trigeminal nerve in numerous ways including the guidance, adhesion and survival of trigeminal nerve fibers.

**Neurotrophins in trigeminal nervous system development**

Neurotrophin-3 may be regulated by RA in *Xenopus laevis*. The neurotrophins are a family of proteins best known for their role in promoting neural growth and cell survival but also have roles in synaptic plasticity and maintenance (Huang and Reichardt 2001; Skaper 2012). Nerve growth factor (NGF) was the first protein of the neurotrophin family discovered (1952) followed by brain-derived neurotrophic factor (BDNF) and neurotrophins 3, 4/5, 6 and 7 (Levi-Montalcini 1987; Huang and Reichardt 2001; Skaper 2012). These proteins are highly conserved and form homodimers with a structure characteristic of other growth factors including transforming growth factor β (TGFβ) and platelet-derived growth factor (PDGF) (Skaper 2012). Neurotrophins are expressed in diverse tissues and cell types including the target tissues of extending axons, mast, Schwann and fibroblast cells, regions being invaded by sensory fibers en route to their targets as well as by some neurons (Huang and Reichardt 2001).

Neurotrophins signal through two distinct classes of receptors: tropomyosin-related kinase (Trk) and p75 of the tumor necrosis factor receptor superfamily (Huang and Reichardt 2001; Skaper 2012). Trk receptors, of which there are three, TrkA, B, and C, are tyrosine receptor kinases and have differing binding affinities for the various neurotrophin ligands. NGF binds TrkA with high affinity, BDNF and NT-4/5 bind TrkB with high affinity and NT-3 binds...
TrkC with high affinity in mammals (Huang, Wilkinson et al. 1999). NT-3 also binds TrkA and B, but with a lower affinity (Skaper 2012). The p75 receptor binds all neurotrophins with an equally low affinity (Skaper 2012). Signaling through Trk receptors promotes cell survival and axonal outgrowth by acting upon diverse signaling pathways. For example, cell survival is promoted by the Akt/protein kinase B pathway (Huang and Reichardt 2001; Skaper 2012). Signaling through p75 can initiate several opposing cellular events including apoptosis, promoting neural outgrowth and facilitating ligand binding to Trk receptors (Bamji, Majdan et al. 1998; Salehi, Roux et al. 2000; Skaper 2012).

Neurotrophins have been implicated in the development of the trigeminal nerve system in all species studied (Arumae, Pirvola et al. 1993; Ibanez, Ernfors et al. 1993; Muragaki, Timothy et al. 1995; Williams, Backstrom et al. 1995; Islam, Gagnon et al. 1996; Wilkinson, Farinas et al. 1996; Quartu, Geic et al. 1997; Huang, Wilkinson et al. 1999; Huang, Dorey et al. 2007). In *Xenopus* it has been shown that BDNF in the cement gland acts as a target-derived cue for the mandibular branch of the trigeminal nerve and that knockdown of BDNF results in misguided mandibular nerves (Huang, Dorey et al. 2007). Knockdown of either NT-3 or TrkC has been shown to result in the death of cells in the trigeminal ganglia (Huang, Wilkinson et al. 1999). Trk receptors are expressed in the trigeminal ganglia of mouse, rat, chick, human and zebrafish (Williams, Backstrom et al. 1995; Quartu, Geic et al. 1997; Huang, Wilkinson et al. 1999; Pan, Choy et al. 2012). Only TrkB expression has been analyzed in *Xenopus*, where its transcripts are found in the trigeminal ganglia and along the trigeminal nerve (Islam, Gagnon et al. 1996).
In conclusion, neurotrophins and their receptors are good candidates for promoting guidance and survival of the trigeminal nervous system. It is possible that neurotrophins play a similar survival promoting role in *Xenopus*.

**Semaphorins in cranial nervous system development**

RA may regulate axon guidance by regulating Semaphorins. Semaphorins are a diverse yet highly conserved class of molecules best known for their role in neural development and axon guidance. Semaphorins have been implicated in cell adhesion, cancer, coordination of immune response, and angiogenesis among other functions (reviewed in (Yazdani and Terman 2006)). All Semaphorin family members have: 1) a β propeller topology, a conformation common to proteins with diverse functions and 2) a 500 amino acid Sema domain at the amino terminus (reviewed in (Nakamura, Kalb et al. 2000; Yazdani and Terman 2006)). The Semaphorin family is divided into 8 classes, 1-7 and V (viral) (reviewed in (Yazdani and Terman 2006)). Semaphorins are expressed in all metazoans studied and dynamically expressed during development, yet no particular expression pattern defines any one class (reviewed in (Yazdani and Terman 2006)). In the development of the nervous system, Semaphorins signal through Plexin receptors while the Neuropilins act as coreceptors for class 3 Semaphorins (Takahashi, Fournier et al. 1999; Nakamura, Kalb et al. 2000; Yazdani and Terman 2006).

Semaphorins are known to function as attractants and repellents in axon guidance and the migration of neural crest cells, neuroblasts, glia and neurons (Chilton and Guthrie 2003; Kantor, Chivatakarn et al. 2004; Yazdani and Terman 2006; Huettl and Huber 2011; Maier, Jolicoeur et
al. 2011). Both Semaphorin 3A and 3F have been shown to be required for channeling the neural crest derived from rhombomeres 2 and 4 and keeping the streams separate (Schwarz, Vieira et al. 2008). In Neuropilin 1 and 2 double knockout mice the individual trigeminal nerves becomes defasciculated yet most fibers make it to the proper target area (Schwarz, Vieira et al. 2008). This indicates that Semaphorins 3A and 3F, the ligands of Neuropilins 1 and 2 respectively, are important for the condensation of the ganglia and therefore proper organization of the cranial ganglia and nerves, but do not directly affect the guidance of these nerves (Schwarz, Vieira et al. 2008).

Semaphorin 4B is expressed in the cranial region of *Xenopus* and is therefore a good candidate gene that may influence trigeminal development (Koestner, Shnitsar et al. 2008). Further evidence that Sema4B may be important in the guidance of the trigeminal nerves comes from zebrafish studies. Knockdown of Plexin receptors resulted in misguidance of trigeminal fibers. This data supports a hypothesis whereby trigeminal neurons expressing Plexins are influenced by the orofacial environment expressing Sema4B (Tanaka, Maeda et al. 2007).

In summary semaphorins are good candidates for regulation of trigeminal or cranial nerve development.

*Activated leukocyte cell adhesion molecule (ALCAM)*

RA may also be important for regulating cell adhesion. Activated leukocyte cell adhesion molecule (ALCAM) is a transmembrane glycoprotein belonging to a subgroup of proteins in the immunoglobulin superfamily. It is characterized by five extracellular immunoglobulin (Ig)
domains: two amino-terminal, variable-type domains and three constant-type Ig folds; as well as a transmembrane region and a short cytoplasmic tail. The other two proteins of this group are CD146/MUC18 and B-CAM/Lutheran (basal cell adhesion molecule/Lutheran blood group antigen) (Swart 2002). ALCAM was first identified by its interaction with CD6, a SRCR type I cell surface protein expressed on cells of the immune system but has since been shown to form homophilic bonds (Whitney, Starling et al. 1995; van Kempen, Nelissen et al. 2001; Swart 2002).

ALCAM has been shown to be imported for the proper development of the nervous system in multiple model systems (DeBernardo and Chang 1996; Weiner, Koo et al. 2004; Diekmann and Stuermer 2009). Among its various functions in development ALCAM has been implicated in promoting the fasciculation of both sensory and motor nerves (Weiner, Koo et al. 2004; Diekmann and Stuermer 2009). Knockout studies in mice and knockdown studies in zebrafish of ALCAM result in retinal nerve and thoracic motor nerve defasciculation respectively. In addition, ALCAM has been shown to be expressed in the profundal and trigeminal ganglia of *Xenopus* (Gessert, Maurus et al. 2008; Gessert and Kuhl 2009). It is therefore possible that ALCAM is also functioning to bundle the trigeminal fibers and maintain their fasciculation in *Xenopus laevis*.

Trigeminal nerve development requires the interaction of multiple families of proteins and signaling molecules. Here we propose that RA regulates a subset of such proteins. Components of the retinoic acid signaling pathway are expressed in the region innervated by the
trigeminal nervous system including RALDH2 and RARγ in *Xenopus* and may influence the expression of genes necessary for the proper development of the trigeminal nerve. We have identified three genes: NT-3, Sema4B and ALCAM as candidate regulators of trigeminal nerve development. Therefore, the aims of this research are to begin to assess the role that RA and these candidate regulators play in trigeminal nerve development using the model organism *Xenopus laevis*. The specific goals of this research are outlined below:

1. To characterize the development of cranial innervation in *Xenopus laevis*

2. To determine the effect of RA signaling on trigeminal nerve development by chemical inhibition of RAR

3. To begin to identify candidate regulators of trigeminal nerve development whose transcription is influenced by RA signaling
Experimental Approach

In the present study, the role of retinoic acid in the development of the trigeminal nervous system, specifically the mandibular branch, is examined using the model organism *Xenopus laevis*. *Xenopus* is an ideal tool to use to study trigeminal nerve development since it has been used as a model of trigeminal nervous system development and function for over a quarter century (Roberts and Blight 1975; Davies, Kitson et al. 1982; Davies 1988; Boothby and Roberts 1992; Honore and Hemmati-Brivanlou 1996; Huang, Dorey et al. 2007). In addition, *Xenopus* is amenable to embryological studies since their eggs are large, development is entirely *ex utero* and many embryos can be easily and inexpensively obtained.

Previous studies of retinoic acid’s effect on the cranial nervous system focus mostly on the posterior cranial nerves. Studies that do focus on trigeminal nervous system development are limited to the vitamin-A deficient (VAD) rat model and indicate a role for RA in forming connections between the trigeminal ganglia and the hindbrain (Clagett-Dame, McNeill et al. 2006). This work is the first to examine the role that RA plays in the development of the peripheral projections of the trigeminal nerve and the first to implicate RA in the bundling of the trigeminal nerve fibers.

Cranial nerve development was assessed in *Xenopus laevis* using an antibody raised to a neurofilament associated protein. To examine RA signaling in trigeminal nerve development the synthetic retinoid BMS-453 was used to inhibit retinoic acid receptors (RARs). *Xenopus*
embryos were treated with BMS-453 from stage 18/19 (20-21hpf) until stage 37/38 (53hpf) during a period of development encompassing initial trigeminal nerve outgrowth and target innervation. The effects of RAR inhibition were assessed using 6G7, an antibody raised against β tubulin combined with confocal microscopy. Unbundling of the trigeminal nerve in the region of the eye was observed in BMS-453 treated embryos. To begin to understand how RA influences the development of the trigeminal nervous system in situ hybridization technique combined with qRT-PCR was used to analyze the expression of genes previously identified by our lab as downregulated in response to RAR inhibition including neurotrophin-3 (NT-3), Semaphorin 4B (Sema4B) and activated leukocyte cell adhesion molecule (ALCAM). Future experiments need to be conducted to further analyze the function of these genes in trigeminal nervous system development.
Materials and Methods

Animals

The methods of Sive et al. (2000) were used to obtain and culture *Xenopus laevis* embryos. Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop 1967).

Immunochemistry

Embryos were fixed in 4% paraformaldehyde (PFA) overnight at 4°C, followed by three one hour washes in phosphate buffer saline with Tween (PBT) and stored at 4°C. Embryos were incubated for one day at 4°C in either primary monoclonal antibody raised to neurofilament associated protein (DSHB, 3A10 diluted 1:500 in PBT) or an antibody raised to β-tubulin (DSHB, 6G7 diluted 1:100 in PBT). Following three one hour washes in PBT embryos were incubated in secondary antibody for one day. Secondary antibodies were goat-anti-mouse IgG Alexa Fluor 488 and goat-anti-mouse IgG Alexa Fluor 568 (Invitrogen), diluted 1:500 in PBT. Counterstains were propidium iodide (Sigma, P4864 diluted 1:1000 in PBT) or Phalloidin (Invitrogen, A12379 diluted 1:50 in PBT) and were added with the secondary antibody. Embryos were mounted in 90% glycerol and analyzed by confocal microscopy using a Nikon Eclipse E600 microscope and Nikon EZ C1 Gold Version 3.80 software.
Immunohistochemistry optimization

The staining protocol for 3A10 was modified from Dickinson and Sive (2006) (above) to reduce background staining. Sodium borohydride (NaBH₄) was used as described (Wallingford 2010) to reduce autofluorescence. NaBH₄ was made in a 100mM solution (3.784g NaBH₄/1000mL phosphate buffer saline, PBS) and allowed to sit 48 hours at 4°C. Embryos were bathed in NaBH₄ in culture plates for 4 hours at room temperature followed by three one-hour washes prior to the addition of primary antibody. For those embryos receiving blocking, solution primary 3A10 antibody was diluted in blocking solution (5% bovine serum albumin, BSA, in PBT and normal goat serum, at a ratio of 10:1) immediately before it was applied to the embryos. Embryos were mounted in 90% glycerol and analyzed using confocal microscopy.

Inhibitor treatments

Retinoic acid receptor inhibitor, BMS-453 (Tocris (3409) 10mM in DMSO, stock solution) was applied to embryos at a concentration of 20μM (4μL stock solution in 2mL 0.1x MBS, modified Barth’s saline, pH 7.8) to inhibit retinoic acid signaling. Dimethyl sulfoxide (DMSO) was added at a concentration of 1% (15μL and 20μL in 2mL 0.1x MBS for inhibitor treated and control embryos respectively) to facilitate the entry of BMS-453 into the embryos. Chemical treatments were conducted in 12- or 24-well plates with 4-5 embryos per well and wrapped in aluminum foil to avoid inactivation of the light sensitive BMS-453. Inhibitor treatments began at stage 18/19 (20-21hpf), just after neural tube closure and ended at stage
37/38 (53hpf) when embryos were fixed in 4% PFA. For analysis using qRT-PCR, treatments were ended at stage 34 (44hpf) when embryos were prepared for Trizol extraction (see qRT-PCR below).

**Whole mount in situ hybridization**

In situ hybridizations were performed following the protocol of Sive et al. (2000) omitting the proteinase K treatment. Plasmids containing probes for *NT-3* (BC074327.1, from Openbiosystems, MXL1736-9508938) and *Sema4B* (BC077964.1, from Openbiosystems, MXL1736-9507154) were obtained already transfected into *E.coli* cells in glycerol. Plasmid containing *E. coli* cells were grown in Luria Bertani (LB) broth with 2% ampicillin overnight at 37°C. Plasmids were isolated and purified using the QIAprep® Spin Miniprep Kit (Qiagen). Plasmids were linearized using restriction digest enzymes from New England Biolabs and appropriate buffers at 37°C for 5 hours. Linearized plasmids were run on a 0.8% agarose gel (SeaKem® GTG® Agarose) with 1% ethidium bromide (Millipore, 4410-OP) for purification. Gel extraction was performed using the QIAquick® Gel Extraction Kit (Qiagen) followed by a transcription reaction to obtain a digoxigenin (DIG)-labeled RNA probe.

Embryos were dechorionated with forceps and fixed in 4% PFA overnight. After three washes in PBT to remove fixative, embryos were dehydrated in methanol series and stored at -20°C overnight. The next day embryos were rehydrated from methanol with PBT and washed in 0.1M triethanolamine (pH 7.8). Embryos were bleached until white in a solution of hydrogen
peroxide, 2x saline-sodium citrate (SSC) buffer and formamide (10:9:1 ratio respectively) on a
light box. Once bleached, embryos were transferred to microcentrifuge tubes, bathed in
hybridization buffer, and incubated overnight at 60°C. RNA probe was added at a concentration
of 4µg/mL and allowed to incubate for 24 hours. After the incubation period excess probe was
washed out with SSC buffer and maleic acid buffer (MAB) and embryos were incubated in an in
situ blocking solution (1 Boehringer Mannheim Blocking reagent (BMB): 1 lamb serum: 3
MAB) overnight. Anti-DIG-Alkaline phosphatase (AP) antibody was used at a concentration of
0.63µL per mL of blocking solution and allowed to incubate overnight. Anti-DIG-AP was
washed out with MAB and AP buffer. The colormetric reaction was performed using 56µL NBT
and 28µL BCIP diluted in 10mL of AP buffer. The colormetric reaction was stopped by washout
of the NBT/BCIP solution with PBT and fixation with 4% PFA.

qRT-PCR

Tissue was collected from embryo heads removed from BMS-453 (RAR inhibitor)
treatment at stage 34 (44hpf) and from stage 34 control embryos. Twenty embryo heads were
collected for each treatment group and stored in Trizol (Invitrogen) at -20°C. Total RNA was
isolated using Trizol extraction followed by a lithium chloride solution (Ambion) precipitation.
cDNA was prepared using the Omniscript Kit (Qiagen) and qRT-PCR was performed using
SYBR Green (Qiagen) and a BIO RAD CFX96 Real-Time System (C1000 Touch Thermal
Cycler chassis). ALCAM primer sequences were GAAGGTGATAACATCACTCTCCAA and ATAAATCCCCAGTTGCATTTCG.
Results

Time course of Xenopus cranial nerve development revealed by immunohistochemistry

To date there have been no extensive studies of the development of the cranial nerves in *Xenopus laevis* outside of a few studies focusing on the trigeminal nerve, specifically its mandibular branch (Davies, Kitson et al. 1982; Honore and Hemmati-Brivanlou 1996; Huang, Dorey et al. 2007). These studies do not provide enough detail of the other branches or the other nerves to make an accurate assessment of their normal organization. Though the work of Nieuwkoop and Faber (1967) provide a written account descriptive enough to identify the cranial nerves, it is not sufficient to outline the developmental trajectory of the nerves in detail. In order to understand how retinoic acid is involved in the development of the cranial nerves I performed a developmental time course to visualize the unperturbed trajectory of the nerves.

To visualize developing axon tracts in embryos of *Xenopus laevis* the neurofilament-associated protein antibody, 3A10, was used. This antibody, although developed for use in chick, has been shown to work in *Xenopus* by previous studies and my own antibody control experiments (Supp Fig 1) (Schuff, Rossner et al. 2007). Embryo nerves were labeled at various stages corresponding to events in neural development as described by Nieuwkoop and Faber (1967). At stage 28 (32hpf) (Fig 1A) the trigeminal nerve is visible extending from the trigeminal ganglia, just posterior to the eye, and is beginning to innervate its embryonic target, the cement gland. The absence of 3A10 labeling posterior to the trigeminal nerve is in accordance with previous data describing an anterior-to-posterior development of the cranial
nerves (Nieuwkoop 1967; Cox, Lamora et al. 2011). At stage 34 (44hpf) (Fig 1B) there is extensive arborization of the mandibular branch of the trigeminal nerve in the cement gland in addition to branching of the ophthalmic branch of the trigeminal nerve dorsal and anterior to the eye in the head skin. In addition, the terminals of the facial nerve are visible just posterior to the mandibular nerve in the hyoid arch, the embryonic target of the facial nerve (Nieuwkoop 1967; Schlosser and Roth 1995). Also at stage 34 (44hpf) (Fig 1B) the glossopharyngeal nerve (IX) and vagus nerve (X) are visible extending ventrally (Lin and Szaro 1996). From stage 37 (53hpf) to stage 43 (87hpf) (Fig 1C and 1D) there is further branching and extension of the trigeminal, facial, glossopharyngeal and vagus nerves. At stage 37 the last of the epibranchial placodes, those placodes contributing cells to the facial (VII), glossopharyngeal (IX) and vagal (X) ganglia, have disappeared (Nieuwkoop 1967; Schlosser and Northcutt 2000).

Development of the cranial nervous system in Xenopus is in accord with previous descriptions by Nieuwkoop and Faber (1967). The antibody 3A10 labeling was found in the trigeminal (V), facial (VII), glossopharyngeal (IX) and vagus (X) nerves. The trigeminal nerve is the first to appear and innervate its target and takes the stereotypical course from the trigeminal ganglia to the cement gland as previously described (Nieuwkoop 1967; Davies, Kitson et al. 1982; Honore and Hemmati-Brivanlou 1996). With the development of the trigeminal nervous system assessed in control embryos the effect of RAR inhibition on trigeminal nerve development was examined.


**Antibody optimization**

For visualizing cranial nerve development in *Xenopus* embryos the staining protocol for 3A10 was modified from Dickinson and Sive (2006) to reduce background fluorescence potentially caused by nonspecific binding of the primary antibody (3A10) or by the auto fluorescence of yolk proteins (Wallingford 2010). *Xenopus* yolk proteins are known to auto fluoresce and are numerous and densely packed in younger embryos and become less abundant as they are metabolized and the embryo grows (Wallingford 2010). To reduce auto fluorescence the reducing agent sodium borohydride (NaBH₄) was used following the protocol of Wallingford (2010). To reduce nonspecific staining 3A10 was diluted in a blocking solution (5% bovine serum albumin in PBT and normal goat serum, ratio of 10:1) in addition to decreasing concentrations of primary antibody. The primary antibody incubation time was also reduced to prevent background staining (see **Supp Table 3** for experiment outline).

From this optimization experiment it was determined that reducing the concentration of 3A10 from 1:100 to 1:500 and decreasing the time the embryos were incubated in 3A10 from two to one day reduced background staining (**Fig 2**). Treatments with NaBH₄ and blocking solution were not effective at improving 3A10 staining and therefore I did not use these in my experiments.
**Decreased retinoic acid signaling results in defasciculation of the trigeminal nerve in the eye region**

Since retinoic acid has been shown to influence the morphogenesis of the orofacial region, it was hypothesized that retinoic acid signaling influences the development of the cranial nervous system in this area. Retinoic acid has been shown to alter structures of the first pharyngeal arch (Dupe and Pellerin 2009). Since the mandibular branch of the trigeminal nerve is known as the nerve of the first pharyngeal arch it was a candidate for analysis (Cox, Lamora et al. 2011). To observe how retinoic acid influences mandibular nerve development in *Xenopus*, embryos treated with the RAR inhibitor, BMS-453, were compared to control embryos and analyzed using the anti-β tubulin antibody 6G7 (Fig 3). The β-tubulin antibody was used to label cranial nerves since it is a more comprehensive neuronal marker and the antibody raised against the neurofilament associated protein does not label the entire cranial peripheral system (Honore and Hemmati-Brivanlou 1996). Mandibular nerves of inhibitor treated embryos followed the expected tract and formed terminal arborizations in the cement gland as in control embryos. However, mandibular nerves of inhibitor treated embryos were significantly more defasciculated in the region around the eye than mandibular nerves of controls (Fig 4) (42% of length defasciculated in treated versus 2% of length defasciculated in control embryos, 2 experiments, n=6 for each group, p-value= 0.0130 using Student’s t-test). In order to assess the fasciculation of trigeminal nerve fibers I established parameters to define fasciculation.
Establishing parameters to define fasciculation

Fasciculation was defined based on the thickness of the largest nerve tract and number of branches leaving the nerve tract. Measurements were performed on confocal images taken at 20x magnification using the Nikon Elements software where one pixel equals 0.323 microns (Fig 5 A and B). Defasciculated regions of both control and BMS-453 (RAR inhibitor) treated mandibular nerves were significantly thinner than fasciculated regions of corresponding control and BMS-453 treated mandibular nerves (Fig 6) (Control: Fasciculated thickness = 5.7µm, Defasciculated thickness = 1.17µm; BMS: Fasciculated thickness 4.11µm, Defasciculated thickness= 1.33µm) (2 experiments, n=6 for both groups, p-value= 0.0282 for control and p-value= 0.0023 for BMS-453, Student’s t-test). There was no significant difference in overall thickness of the mandibular nerve between control and BMS-453 treated embryos (Supp Fig 3) (Control average thickness= 5.10µm; BMS average thickness= 3.20µm; p-value= 0.0808, n= 6 for both groups, Student’s t-test). There was also a significant increase in the number of branches observed leaving the main tract of the trigeminal nerve in defasciculated regions of control and BMS-453 treated embryos compared to fasciculated regions (Fig 6) (Control: # of branches per % length of fasciculated nerve= 1.19, # of branches per % length of defasciculated nerve = 72.46; BMS: # of branches per % length of fasciculated nerve= 1.14, # of branches per % length of defasciculated nerve = 16.91) (2 experiments, n=6 for both groups, p-value= 0.0306 for control and p-value= 0.0420 for BMS-453, Student’s t-test).
Inhibition of RA signaling in the orofacial region of *Xenopus* embryos results in an unbundling, or defasciculation, of the mandibular branch of the trigeminal nerve without interfering with the guidance of this tract. Defasciculation was quantified based on two parameters: thickness of the nerve fibers and number of branches leaving the main nerve tract. Within both control and RAR inhibitor treated embryos defasciculated regions were both thinner than fasciculated regions and had more branches emanating from the main tract in defasciculation regions compared to fasciculated regions. Since RA has been shown to alter structures derived from the first pharyngeal arch (reviewed in (Duester 2008)), the area innervated by the trigeminal nerve, it was of interest to determine if the defects in nerve development could be caused by a malformed environment. Therefore, I examined changes in the eye and trigeminal nerve length in this region.

*Retinoic acid receptor inhibitor treatment does not change eye diameter or trigeminal nerve length*

Since retinoic acid is known to influence the development of the eye it was of interest to determine the eye diameter as well as the length of the trigeminal nerve in the region around the eye to ensure that comparisons of fasciculation could be made between control and BMS-453 (RAR inhibitor) treated embryos. According to previous literature, inhibition of retinoic acid signaling should lead to smaller eyes (Duester 2008; Kennedy and Dickinson 2012; Le, Dowling et al. 2012). However, my data show that there was no significant difference in eye diameter between control and BMS-453 treated embryos *(Fig 5C)* (Control eye diameter= 147µm; BMS
eye diameter= 163µm, $p$-value 0.2803, Student’s $t$-test). This could be due to several factors including the RAR inhibitor treatment time. Perhaps eye diameter in the dorsal-ventral axis has been specified before inhibitor treatment is initiated.

In addition, the length of the trigeminal nerve in the region of the eye was analyzed to further ensure that comparisons could be made between control and treated embryos. There was no significant difference in the length of the trigeminal nerve in the eye region of control and treated embryos (Fig 5D) (Control length= 165µm; BMS length= 185µm; $p$-value= 0.1168, Student’s $t$-test). Therefore, it appears that inhibition of RARs at the time point of development examined does not interfere with dorsoventral patterning of the eye or trigeminal nerve length. The effect of RAR inhibition on neural crest cells was examined since these cell give rise to the trigeminal ganglia.

Retinoic acid regulates the fasciculation of the trigeminal nerve independent of its effects on cranial neural crest

Inhibition of RA signaling began at stage 18/19 (20-21hpf) just prior to neural tube closure and before neural crest migration and specification at stage 21 (22hpf). Since neural crest cells have been shown to be important in organizing the condensation of the cranial ganglia it was of interest to determine whether RAR inhibition had an effect on cranial neural crest migration (Begbie and Graham 2001; McCabe and Bronner-Fraser 2009; Blentic, Chambers et al. 2011). Neural crest migration was assessed using the marker $AP$-2 and no difference in expression pattern was found in embryos deficient in retinoic acid signaling (Fig 7). All four
streams of cranial neural crest are visible in both control and RAR inhibitor treated embryos (Smith, Robinson et al. 1997). In addition, the neural crest streams in RAR inhibitor treated embryos are not diminished in size and have migrated ventrally to the same extent as in control embryos. This indicates that neural crest specification and migration were not delayed or perturbed by RAR inhibition. This is in accord with a previous treatment of BMS-453 performed in our lab commencing at stage 24 (26hpf) after neural crest began migrating and after the trigeminal ganglia begins condensing. In addition, this finding is supported by mouse data indicating that RARγ/β knockout in cranial neural crest cells lineages does not disrupt migration or specification (Dupe and Pellerin 2009). Therefore, it is unlikely that trigeminal nerve defasciculation is caused by misguided or absent neural crest cells.

**Analysis of candidate regulators of trigeminal nerve development**

In order to understand how retinoic acid is influencing the fasciculation of the trigeminal nerve, we analyzed gene expression data previously conducted by our lab looking for neural genes that are downregulated when RAR is inhibited. Microarray analysis was conducted using Affymetrix chip and tissue collected from the orofacial region of embryos at stage 30 (35hpf) at the end of RAR inhibitor treatment or control treatment. We identified several known neural genes including neurotrophin-3 (NT-3) and semaphorin 4B (Sema4B) as well as the cell adhesion molecule ALCAM (activated leukocyte cell adhesion molecule). To determine whether these genes are expressed at the appropriate time and place to influence trigeminal nerve
development, I performed \textit{in situ} hybridization for NT-3 and Sema4B as well as using qRT-PCR to analyze ALCAM expression.

\textbf{Expression pattern of Neurotrophin-3}

The spatial expression pattern of \textit{NT-3} in the cranial region was examined using \textit{in situ} hybridization. From stage 22/23 (24hpf) through stage 35 (50hpf) \textit{NT-3} transcripts were found in the eye and otic vesicle (\textbf{Fig 8A-C}). At stage 35 (50hpf) \textit{NT-3} transcripts are detectable in and ventral and posterior to the maxillary process (\textbf{Fig 8C}). These \textit{in situ} data are in accordance with \textit{NT-3} expression data in mouse where it has been shown to be expressed in the maxillary process and eye (Wilkinson, Farinas et al. 1996; Farinas, Wilkinson et al. 1998; Bennett, Zeiler et al. 1999). Therefore, \textit{NT-3} is expressed at a time and place that could influence the trigeminal nervous system development.

\textbf{Expression pattern of Semaphorin 4B}

The spatial expression pattern of \textit{Sema4B} in the cranial region was examined using \textit{in situ} hybridization. From stage 22/23 (24hpf) (\textbf{Fig 9A}) through stage 33/34 (44hpf) (\textbf{Fig 9B}) \textit{Sema4B} transcripts were found in the cement gland, the lens of the eye, otic placode and in the pharyngeal arches. At stage 33/34 (44hpf) (\textbf{Fig 9B-C}) onwards \textit{Sema4B} transcripts were detectable as a thin line of expression encircling the eye. Expression in the cement gland varied with the stage examined. Early expression of \textit{Sema4B} was detectable in the entire cement gland. As development progressed, \textit{Sema4B} expression in the distal cement gland diminished while proximal expression remained until stage 41 (76hpf) (\textbf{Fig 9C}) at which point \textit{Sema4B} was barely
detectable in the cement gland. Expression in the otic placode was detectable until stage 41 (76hpf). At stage 41 (76hpf), Sema4B was found in the fore- and midbrain regions.

These *in situ* hybridization data is in accordance with a previous study of Sema4B expression in *Xenopus laevis*. Koestner et al (2008) found Sema4B transcripts in the otic placode, cement gland, as well as the migratory neural crest, pronephros and mesoderm. Thus, my data and previous data support a role of Sema4B in the development of the cranial region.

**ALCAM expression analyzed via qRT-PCR**

The temporal regulation of ALCAM by RA in the cranial region was examined using qRT-PCR. Embryos were treated with BMS-453 from stage 18/19 (20-21hpf) until stage 34 (44hpf). qRT-PCR analysis revealed a 2.5-fold decrease in ALCAM expression in the cranial region of RAR inhibitor treated embryos compared to control embryos (Fig 10). ALCAM is expressed in the profundal, trigeminal, anterodorsal lateral line and middle lateral line ganglia as well as the hypoglossal and vagus epibranchial ganglia at this time point of development (Gessert, Maurus et al. 2008). It is possible that this 2.5-fold decrease in ALCAM expression is resulting in reduced trigeminal nerve adhesion and therefore defasciculation of these fibers.

Chemical inhibition of RA signaling results in the defasciculation of the mandibular branch of the trigeminal nerve without affecting the guidance of this nerve. The mechanism behind this remains unclear yet gene expression analysis revealed down regulation of candidate regulators of trigeminal development. Therefore, analysis of the spatial and temporal expression of these genes was performed using *in situ* hybridization technique and qRT-PCR. *In situ*
hybridization data of NT-3 and Sema4B reveal expression in surrounding and target tissues. qRT-PCR analysis of ALCAM shows down regulation of this gene prior to nerve defasciculation. More research is needed to investigate the role of these genes, but this research has shown that these genes are expressed at the correct place and at the correct time to affect trigeminal nerve development.
Discussion

*Immunohistochemical time course of Xenopus cranial nerve development*

In order to analyze the effect of retinoic acid signaling inhibition on the development of the cranial nervous system it was necessary to determine the unperturbed trajectory of the cranial nerves. My analysis of the cranial nervous system of *Xenopus laevis* is mostly in accord with the descriptions of Nieukwoop and Faber (1967). In addition, my observations are supported by other works focusing on the nervous system of *Xenopus* as well as by comparative anatomy (Davies, Kitson et al. 1982; Davies 1988; Honore and Hemmati-Brivanlou 1996; Lin and Szaro 1996; Higashijima, Hotta et al. 2000; Cordes 2001; Huang, Dorey et al. 2007; Tanaka, Maeda et al. 2007; Muller and O'Rahilly 2011; Walsh, Grant et al. 2011). The trigeminal nerve is the first cranial nerve to emerge and innervate its target (*Fig 1A*). The facial nerve is the next to appear at stage 34 (44hpf) (*Fig 1B*). The glosopharyngeal (IX) and vagus (X) nerves appear at stage 34 (44hpf) (*Fig 1B*). The vagus nerve has a distinct inverted “V” shape with a thick root that is evolutionarily conserved in zebrafish and mouse (Cordes 2001; Tanaka, Maeda et al. 2007; Walsh, Grant et al. 2011). This analysis has tracked the development of four of the twelve cranial nerves. Cranial nerves I-IV, VIII, XI and XII were not visible or discernible using the methods employed. Cranial nerves I-IV are obscured by the eye, nerve VIII, the vestibulo-acoustic nerve, is obscured by labeling of the hindbrain behind it and cranial nerves XI and XII are indiscernible from the spinal nerves (Higashijima, Hotta et al. 2000; Cordes 2001). This study is the first to provide a visual description of cranial nervous system development in *Xenopus laevis*. 
**Decreased retinoic acid signaling results in defasciculation of the trigeminal nerve in the eye region**

The formation of the trigeminal nervous system requires the complex interaction of cranial neural crest, ectodermally-derived placodes and neuroectoderm of the hindbrain (Schlosser and Northcutt 2000; Begbie and Graham 2001; Guthrie 2007; Blentic, Chambers et al. 2011). In addition, a diverse array of cell signaling molecules including axon guidance cues, growth factors, cell adhesion molecules and morphogens such as retinoic acid contribute to the development of the trigeminal nerve (Islam, Gagnon et al. 1996; Huang, Wilkinson et al. 1999; Clagett-Dame, McNeill et al. 2006; Huang, Dorey et al. 2007). I have shown that retinoic acid signaling is required for the proper fasciculation of the mandibular branch of the trigeminal nerve in *Xenopus* (Fig 3). Previous work in our lab has shown the RA signaling molecules RALDH2 and RARγ to be expressed in the cranial region at the time of trigeminal outgrowth and target innervation (Kennedy and Dickinson 2012). Therefore, I used the RAR inhibitor, BMS-453, to inhibit RA signaling during trigeminal development. I observed no gross defects of axon guidance in RAR inhibitor treated embryos with trigeminal nerves innervating their embryonic target, the cement gland. However, I observed an unbundling of the fibers of the mandibular branch adjacent to the eye upon termination of treatment at stage 37 (53hpf). It is possible that retinoic acid is needed for proper trigeminal nerve development due to its ability to influence the transcription of key genes involved in either the proper adhesion, guidance or survival of
trigeminal neurons. RA’s effect on trigeminal development could stem from its effect on one or more of these processes.

The influence of retinoic acid on the surrounding tissues

Retinoic acid has been shown to influence the development of structures derived from the first pharyngeal arch (reviewed in (Duester 2008)). This is important since the trigeminal nerve is the nerve of the first pharyngeal arch and perturbations in the surrounding tissues could secondarily cause defects in nerve development. Several lines of evidence from this study and previous studies suggest that the defects in nerve development are not secondary to defects in surrounding tissues. The region of the trigeminal nerve under consideration is adjacent to the eye. Since retinoic acid is also known to be important in eye development and deficiency in RA signaling results in microphthalmia (Dickman, Thaller et al. 1997; Duester 2008) the size of the eye was measured to ensure comparable lengths of trigeminal nerves were assessed in control and RAR inhibitor treated embryos (Fig 5 A). No significant difference in the size of the eye was observed in BMS-453 treated embryos along the dorsal-ventral axis (Fig 5C). In another work using BMS-453 to inhibit RA signaling there was also no observable difference in dorsal-ventral eye diameter (Chen, Pan et al. 2004). Dorsoventral patterning of *Xenopus* eye may occur before our inhibitor treatment begins and is therefore largely unperturbed in our study (Lupo, Liu et al. 2005).

In addition, previous work in our lab has shown BMS-453 treatment to not be toxic during early development since there was no change in apoptotic or proliferating cells prior to
stage 37-40 (53-66hpf). However, at stage 37-40 (53-66hpf) there was a decrease in cells undergoing mitosis (Kennedy and Dickinson 2012). This decrease in cell proliferation may not be attributing to defasciculation of the trigeminal nerve since the ganglia is finished receiving cells from its placode at stage 35 (50hpf) and already has its full complement of ganglion cells before proliferation decreases (Schlosser and Northcutt 2000; Kennedy and Dickinson 2012). Also, defasciculation is observed at stage 37 (53hpf) which may be too soon to result from decreased proliferation beginning at this stage. However, proper axon-environment interactions are required for the guidance and fasciculation of peripheral nerves (Tessier-Lavigne and Goodman 1996; Huettl and Huber 2011; Coate, Raft et al. 2012). The absence of cells bearing adhesion molecules or guidance cues could alter the development of peripheral projections via disrupted axon-environment interactions (Gong and Shipley 1996; Wolman, Sittaramane et al. 2008; Coate, Raft et al. 2012). Cranial neural crest specification and migration in the region of the trigeminal nerve appear unaffected by inhibition of RAR (Fig 7). In addition, no large morphological changes are observed in early development following RAR inhibition (Kennedy and Dickinson 2012). The morphological changes in the first pharyngeal arch that contribute to malformation of the palate and surrounding tissues have been observed to occur beginning at stage 40 (66hpf) (Kennedy and Dickinson 2012). However, we cannot rule this out with our current data and further exploration of the effect of RAR inhibition on the morphogenesis of the surrounding tissues is needed.
**Retinoic acid may be acting through multiple genes to influence the development of the trigeminal nervous system**

Inhibition of retinoic acid receptors results in the defasciculation of trigeminal nerves. Fasciculation of nerve fibers is important in the developing nervous system to ensure that proper connections are made (Van Vactor 1998). Initial pioneer axons navigate axon guidance cues and form connections with target tissues, later developing axons fasciculate along these preexisting axon tracts to navigate to their target (reviewed in (Tessier-Lavigne and Goodman 1996)). Fasciculation of axons simplifies nervous system development (reviewed in (Tessier-Lavigne and Goodman 1996)). To elucidate how deficiency in RA signaling could cause the defasciculation of the trigeminal nerve, we performed gene expression analysis to identify genes downregulated in response to RAR inhibition (Kennedy and Dickinson 2012). We identified several candidate regulators of trigeminal nerve development and selected genes based on their previously identified function or relationship with other family members.

Nerve growth factors and their receptors have been shown in multiple model organisms to play a role in trigeminal nerve development (Wilkinson, Farinas et al. 1996; Huang, Wilkinson et al. 1999; Huang, Dorey et al. 2007; Pan, Choy et al. 2012). Neurotrophin-3 (NT-3) was found to be downregulated in response to RAR inhibition in gene expression analysis previously performed in our lab (ref). My in situ data show that NT-3 is expressed in the eye and later in the tissues ventral and posterior to the eye (Fig 8) just before I observe defects in the trigeminal nerve. This data, in conjunction with previous in situ experiments by Islam et al
(1996) indicating TrkB expression in Xenopus trigeminal ganglia suggests a complementary receptor-ligand expression pattern. In addition, NT-3 has been shown to support the survival of trigeminal neurons by acting directly through TrkB (Huang, Wilkinson et al. 1999). I hypothesize that diffusible NT-3 promotes the survival of trigeminal ganglia cells that express neurotrophin receptors. This hypothesis is further supported by studies in mouse showing that NT-3 or TrkC knockout results in a 70% or 22% loss of neurons compared to wildtype controls (Huang, Wilkinson et al. 1999). Several studies have correlated nerve defasciculation with neuronal apoptosis (Morris, Lin et al. 1999; Newbern, Li et al. 2011). While trigeminal nerves of RAR inhibitor treated embryos do not appear thinner than in controls (Supp Fig 3) it is possible that a small reduction in cell number could result in defasciculation. Whether there is a decrease in neuron number in the trigeminal ganglia of RAR inhibitor treated embryos is a topic currently under investigation in our lab (see Future Directions section below). Thus, I have identified and characterized a potential mechanism by which RA regulates NT-3 in trigeminal nerve development.

Semaphorin 4B (Sema4B), a member of a well characterized family of axon guidance molecules, was also found to be downregulated by RAR inhibition in gene expression analysis performed in our lab (Kennedy and Dickinson 2012). Sema4B is known to cytoplasmically bind a post-synaptic density protein, PSD-95, in vitro and to have a role in synapse formation by regulating the maturation of the post-synaptic density in mouse hippocampus (Burkhardt, Muller et al. 2005; Paradis, Harrar et al. 2007; Shen and Cowan 2010). My in situ data is the first to
show *Sema4B* expression in the lens of the eye as well as in a region that appears to be the
second pharyngeal arch (Fig 9). In zebrafish *Sema3A, Sema3F* and *Sema4E* are detected in the boundary region of the first and second branchial arches where they are required for the fasciculation and correct axonal path finding of the trigeminal and facial primary motoneurons (Xiao, Shoji et al. 2003; Tanaka, Maeda et al. 2007). In addition, Semaphorins 3A and 3G are expressed in the pharyngeal arches of mouse (Sauka-Spengler and Bronner-Fraser 2006; Gammill, Gonzalez et al. 2007). Semaphorins are best known for their ability to inhibit neurite extension, but they can also serve as an attractant (Nakamura, Kalb et al. 2000; Kantor, Chivatakarn et al. 2004; Yazdani and Terman 2006). Based on the expression pattern of Sema4B in *Xenopus*, I hypothesize that it is acting to create an inhibitory surround preventing trigeminal fibers from venturing into the second pharyngeal arch as well as from invading the eye. In addition, *Sema4B* is also expressed in the cement gland. Here it could be serving an inhibitory function to stop neurite outgrowth once contact with the cement gland is established or it could be serving to attract trigeminal fibers to the cement gland. In summary, I have identified and characterized Sema4B as a possible molecule influencing the guidance of the trigeminal nerve.

Axon defasciculation defects are often observed when the expression of cell adhesion molecules is perturbed (Cohen, Taylor et al. 1998; Yu, Huang et al. 2000; Weiner, Koo et al. 2004; Fujita and Nagata 2007). We identified activated leukocyte cell adhesion molecule (ALCAM) as being downregulated in response to RAR inhibition. Gessert et al. (2009) showed *ALCAM* expression in the region of the trigeminal ganglia and axons in *Xenopus*. Therefore, I
designed primers and conducted qRT-PCR for *ALCAM* using cDNA from stage 34 (44hpf) embryos, just prior to the observed defasciculation phenotype, and found that *ALCAM* is downregulated 2.5-fold in the cranial region of BMS-453 treated embryos (Fig 10). Knockdown and knockout studies of ALCAM in zebrafish and mouse result in defasciculation of nerves that express ALCAM including both motor and sensory fibers (Weiner, Koo et al. 2004; Diekmann and Stuermer 2009). Fujita and Nagata (2007) demonstrated that knockdown of another member of the cell adhesion molecule family results in a similar phenotype to what I have observed with RAR inhibitor treatment. Therefore, I hypothesize that RA is influencing the development of the trigeminal nerve by acting through NT-3, Sema4B and/or ALCAM to promote neuronal survival, guidance and/or fasciculation of the mandibular nerve respectively.

Proposed model of retinoic acid signaling in the developing trigeminal nervous system

Based on these results, I have developed a model of the role that retinoic acid signaling plays in regulating the development of the trigeminal nervous system (Fig 11). During normal development, retinoic acid regulates gene expression either directly through genes with retinoic acid response elements or indirectly via non-genomic mechanisms (Duester 2008; Carter, Farrar et al. 2010). In either case, retinoic acid appears to be influencing trigeminal nerve development by influencing gene expression and not by its effect on the morphogenesis of the cranial region and on neural crest. We have identified and began studying three candidate regulators of trigeminal nerve development. It is possible that NT-3 promotes the survival of trigeminal neurons and that down regulation of this gene causes death of neurons and contributes to an
unraveling of the remaining nerve fibers. Sema4B possibly contributes to the guidance of the trigeminal fibers by keeping them on their tract. Deficiency in Sema4B may allow initial pioneering fibers to invade the second pharyngeal arch and eye area causing fibers that follow to go off target. The effect of Sema4B in the cement gland is less obvious since other factors guide the nerve to its target including BDNF (Huang, Dorey et al. 2007). ALCAM expressed in the trigeminal ganglia and nerve may function to promote adhesion among a subset of trigeminal neurons. Deficiency in ALCAM expression could reduce adhesion amongst trigeminal neurons and contributes to defasciculation.

This model is all encompassing yet the defects we observe in trigeminal development could arise from down regulation of only one of the above genes. Alternatively, these proteins could influence the development of the cranial nervous system in other ways. For example, NT-3 could be promoting the survival of nerve fibers innervating the eye or supporting retinal ganglion cells of the eye. At later stages the NT-3 expression domain expands ventrally and posteriorly and it could serve as a target-derived cue for nerve fibers innervating the pharyngeal mesenchyme and epidermis. Changes in the innervation of the eye and epidermis of *Xenopus* were not analyzed in this work. Sema4B could be acting to channel neural crest cells or placode cells of the epibranchial ganglia that give rise to the facial, hypoglossal and vagus nerves. It could also contribute to the guidance and fasciculation of the adjacent facial nerve as does Sema4E in zebrafish. In addition, the strip of ALCAM expression visible in previous *in situ* experiments may be expressed in the environment and by trigeminal axons (Gessert, Maurus et
al. 2008; Gessert and Kuhl 2009). If this is the case, ALCAM could be acting as a surface adhesion molecule promoting the guidance of these fibers by forming a tract of cell adhesion protein for trigeminal fibers to follow. In conclusion, there are many possible ways these RA and these genes can influence cranial nerve development and much work is needed to elucidate the function of each of the genes in nervous system development.
Future Directions

I have shown that retinoic acid is important for the proper development of the trigeminal nerve and that deficiency results in axons of the mandibular branch of the trigeminal nerve becoming defasciculated. The exact mechanism behind how retinoic acid influences trigeminal nerve development is unclear. Therefore, more investigation of retinoic acid regulation of trigeminal nerve development is needed.

Elucidating the function of candidate regulators of trigeminal development

Our lab has shown that NT-3, Sema4B and ALCAM are downregulated in response to RAR inhibition (Kennedy and Dickinson 2012). However, the function of these genes in trigeminal nerve development can only be inferred based upon family relationships and data from other models. Therefore, to determine the function of these genes Morpholino-induced knockdown would need to be performed on each gene individually and the phenotype assessed by immunohistochemistry and in situ markers.

Receptor expression of candidate regulators of trigeminal development

NT-3 receptors are known to be expressed in the trigeminal ganglia in many species, yet only TrkB expression, a low-affinity NT-3 receptor, has been performed in Xenopus (Islam, Gagnon et al. 1996). Analysis of TrkC expression, the high-affinity NT-3 receptor, needs to be performed. TrkC expression could also be assessed after RAR inhibition and its down regulation could further support the hypothesis that NT-3 contributes to the survival of trigeminal ganglia cells expressing relevant receptors. Less is known about Sema4B binding partners. Plexins,
semaphorin receptors, have been shown to be expressed in the trigeminal system of mouse and zebrafish (Perala, Immonen et al. 2005; Tanaka, Maeda et al. 2007). There are sixteen PLEXIN genes (A1-4, B1-4, C1-4 and D1-4) in *Xenopus* and immunoprecipitation assays await appropriate Sema4B and Plexin antibodies.

**Assessing trigeminal development using markers for placode cells and cell death**

*AP-2 in situ* hybridizations indicate that cranial neural crest is not perturbed by RAR inhibition (Fig 7). To ensure that placode cells appropriately contribute to the formation of the ganglia an analogous *in situ* would need to be performed using a probe such as *NeuroD* (Schlosser and Northcutt 2000; Schlosser, Awtry et al. 2008). Likewise, trigeminal ganglia size could be assessed by co-labeling with a pan neural marker and TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) labeling to determine if cell death plays a role in defasciculation.

**Determining whether defects in trigeminal development are secondary to morphological changes in the surrounding tissues**

I have begun to examine the morphology of surrounding tissues to explore the possibility that defects in trigeminal development are caused by morphological changes in the environment. Based on measurements of eye diameter in the dorsoventral plane, trigeminal nerve length as well as assessment of cranial neural crest in the region of the trigeminal nerve it appears that the surrounding tissues are not malformed at the time point examined. However, further work needs to be done to determine the role surrounding tissues play in trigeminal development.
Transplantation experiments transplanting cranial tissue adjacent to the trigeminal system from embryos injected with a dominant negative form of RAR into control embryos could be performed. This would inform us of the effect that surrounding tissues have on trigeminal development independent of the effect that RAR inhibition may have on trigeminal cells. The opposite experiments could also be performed to understand the cell autonomous effects of RAR inhibition on trigeminal ganglia cells. In conclusion, I have only begun to elucidate the function of RA in trigeminal nervous system development and much work is needed to determine the function of genes whose transcription is influenced by RA as well as the influence of surrounding tissues on trigeminal nerve development.
Figures

Cranial Nerve Time Course

At stage 28 (32hpf) A.) the trigeminal nerve (V) is visible extending from its ganglion just dorsal and posterior to the eye to its embryonic target, the cement gland (cg). At stage 34 (44hpf) B.) the facial nerve (VII) is seen innervating the second pharyngeal arch. At stage 37 (53hpf) C.) the facial ganglion is more pronounced (VII) and the vagus nerve (X) is visible. By stage 43 (87hpf) D.) the entirety of the embryonic cranial nervous system is present.

mm- maxillomandibular nerve (branch of the trigeminal), cg- cement gland
The immunohistochemical protocol for 3A10 was modified from the standard protocol of Dickinson and Sive (2006) in order to reduce background staining of the 3A10 antibody. It was determined that reducing the concentration of 3A10 to 1:500 in addition to decreasing the incubation time from two to one day was sufficient to reduce background staining.

V - trigeminal nerve, VII - facial nerve, V1 - ophthalmic branch of trigeminal, mm - maxillomandibular branch of trigeminal, cg - cement gland, np - nasal placode, NaBH₄ - sodium borohydride
Figure 3. Inhibition of retinoic acid signaling results in defasciculation of the mandibular nerve in the eye region.

Comparison of control A-C.) and BMS-453 treated D-E.) embryos at 20x magnification using the anti-β tubulin antibody 6G7 (red) and phalloidin (green) counterstain A, B, D, and F, 6G7 (green) and PI counterstain (red) C and E. The peripheral nervous system around the eye was traced A’-F’ to allow for comparisons. The mandibular nerve of control embryos A-C.) is visible as a continuous fasciculated fiber tract. The mandibular nerve of treated embryos D-E.) is visible as a thin, defasciculated nerve tract that branches extensively in the eye region.

mm- maxillomandibular branch, BMS-453- RAR inhibitor
**Figure 4. The trigeminal nerve is significantly more defasciculated in retinoic acid receptor inhibitor treated embryos compared to controls.**

The trigeminal nerves of embryos were measured as the nerve extended from the trigeminal ganglia until it reached the bottom of the eye. The total length of the nerve as well as the length of the nerve that was determined to be fasciculated was calculated in microns using the Nikon Elements software. The ‘percent fasciculated’ was calculated using the formula (length fasciculated)/(total nerve length). Embryos were taken from two experiments with n= 6 for both groups. Control nerves were significantly more fasciculated (98%) than treated nerves (58%). (*p*-value = 0.0130, Student’s *t*-test)
Eye and Nerve Measurements

Figure 5. Measurements and comparisons of eye diameter and trigeminal nerve length in the eye region in control and inhibitor treated embryos.

Trigeminal nerve length was measured A-B.) in control and BMS-453 treated embryos in the region around the eye using the Nikon Elements software. Regions determined to be defasciculated and fasciculated were measured and the percent length defasciculated was calculated for each embryos using the formula (length defasciculated)/(total length). All images considered for analysis were taken at 20x where 1 pixel= 0.323 µm. The eye diameter C.) of control and BMS-453 treated embryos was measured to ensure similar regions were compared in the two groups. There was no significant difference in eye diameter of control versus BMS-453 treated embryos. (2 experiments, n=6 for both groups, p-value= 0.2803, Student’s t-test). The length of the trigeminal nerve D.) in control and BMS-453 treated embryos was compared to ensure that comparisons regarding percentage fasciculation could be made. There was no significant difference in average trigeminal nerve length in the region of the eye of control versus BMS-453 treated embryos. (2 experiments, n=6 for both groups, p-value= 0.1168, Student’s t-test). Tubulin (red), Phalloidin (green).

Defas- Defasciculated, Fas- Fasciculated
Figure 6. Nerve thickness and number of branches can be used to define fasciculation

The thickness A.) of regions considered to be fasciculated and regions considered to be defasciculated was measured using the Nikon Elements software. Relative thicknesses of these regions were compared within individual nerves since there was much variation in nerve thickness amongst individual embryos. Based on these measurements and separate Student’s t-tests for each group it was found that regions considered to be defasciculated were significantly thinner than regions considered to be fasciculated (Control: p-value= 0.0282, BMS-453: p-value= 0.0023) and that this definition of fasciculation can be used in control as well as treated embryos. The number of branches B.) diverging from the main (thickest) fiber tract in regions considered to be fasciculated and regions considered to be defasciculated was measured using the Nikon Elements software. Based on these measurements and separate Student’s t-tests for each group it was found that regions considered to be defasciculated had significantly more branches diverging from the main tract (Control: p-value= 0.0306, BMS-453: p-value= 0.0420) and that this definition of fasciculation can be used in control as well as treated embryos.
Figure 7. Specification and migration of cranial neural crest cells as assessed by AP-2 in situ hybridization.

The specification and migration of cranial neural crest was assessed at stage 34 (44hpf) using the neural crest marker AP-2. No obvious differences were observed between the expression pattern observed in control embryos A.) compared with that of BMS-453 treated embryos B.).

cg- cement gland, n1-4- neural crest streams 1-4
Figure 8. Neurotrophin-3 in situ hybridization time course.

Neurotrophin-3 expression is visible at stage 23 (24hpf) A.) in the eye region. At stage 29 (35hpf) B.) NT-3 transcripts persist in the eye. By stage 35 (50hpf) C.) NT-3 expression is still found in the eye region as well as tissue ventral and posterior to the eye. Sense control embryos A’, B’, and C’) do not exhibit any labeling.

cg- cement gland
Figure 9. Semaphorin 4B in situ hybridization time course.
Semaphorin 4B expression is visible at stage 22/23 (24hpf) A.) in the otic vesicle, cement gland and in the pharyngeal arches. At stage 31 (37hpf) B.) Sema4B transcripts are not found in the distal cement gland. The stripe of expression in the pharyngeal arches has expanded dorsally and expression is visible in the lens of the eye. At stage 33/34 (44hpf) C.) the expression pattern resembles that of stage 31 with further recession of Sema4B from the distal cement gland. By stage 41 (76hpf) D.) Sema4B transcripts are no longer visible in the otic vesicle, cement gland, or along the border of the first and second pharyngeal arch. However, expression is found in the brain.

cg- cement gland, ph- pharyngeal arch border, ot- otic vesicle
mRNA was extracted from whole heads from control and BMS-453 treated embryos stage 34 (44hpf) using Trizol and converted to cDNA using the Omniscript kit from Qiagen. At stage 34 ALCAM transcripts are down regulated in the cranial region of embryos treated with BMS-453 by 2.5-fold. This down regulation occurs 9 hours before the observed defasciculation defect at stage 37 (53hpf). (p-value= 0.0066, Student’s t-test).
Figure 11. Model of retinoic acid signaling in the development of the mandibular branch of the trigeminal nerve.

In situ data that I have conducted on Sema4B and NT-3 in conjunction with previous works showing Trk receptor expression and ALCAM expression in the Xenopus trigeminal nerve have allowed me to propose a model for the role of these genes in trigeminal nerve development A and B). NT-3 expressed in the eye diffuses to Trk expressing trigeminal neurons promoting their survival. Semaphorin 4B, found on either side of the mandibular nerve tract, is possibly forming an inhibitory surround keeping the mandibular nerve on track. Gessert et al (2009) have shown ALCAM expression in the trigeminal ganglia of Xenopus. Since this gene is down regulated 2.5-fold in BMS-453 treated embryos and previous works in zebrafish and mouse show that knockdown of this gene causes defasciculation, it is possibly that ALCAM is serving to promote cell adhesion amongst mandibular nerve fibers. When these genes are knockdown C) trigeminal neurons could die, causing an unraveling of the nerve resulting in defasciculation. Reduced inhibitory signal from Sema4B could results in fibers becoming “lost” and invading tissue they otherwise would not. Reduced cell adhesion protein could directly result in defasciculation by reducing the adherence of nerve fibers to one another.
Literature Cited


**Supplemental Figure 1. Horizontal sections of adult Xenopus laevis brain labeled with 3A10.**

All images are oriented such that median is left and lateral is right. **A-C.** are confocal images of increasing magnification of brain slices stained with 3A10 (1:500 in PBT) and Alexa Fluor 488 (1:500 in PBT) showing that 3A10 labels neuronal processes. **D-F.** are confocal images of adjacent slices of brain labeled only with the secondary Alexa Fluor 488 showing that the staining in **A-C.** is specific. All images are counterstained with propidium iodide.
Supplemental Figure 2. Eye measurements in control and retinoic acid inhibitor treated embryos.
Since retinoic acid is known to influence eye development the diameter of the eyes in control and BMS-453 were measured as shown above (vertical bar) using the Nikon Elements software. The portion of the trigeminal nerve within the horizontal bars was examined for fasciculation. All images considered for analysis were taken at 20x where 1 pixel = 0.323 µm. Tubulin (red), Phalloidin (green).
V - trigeminal nerve
Supplemental Figure 3. Average trigeminal nerve thickness in control versus retinoic acid receptor inhibitor treated embryos.

Trigeminal nerve thickness was measured in control and BMS-453 treated embryos using the Nikon Elements software. Individual nerves were measured in three locations and these measurements were averaged to account for variability in thickness within individual embryos. All images considered for analysis were taken at 20x where 1 pixel = 0.323 µm. (2 experiments, n=6 for both groups, p-value = 0.0808, Student’s t-test)
Supplemental Table 1: Summary of the cranial nerve number and function

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**Supplemental Table 3: Antibody optimization experiment outline**

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Notes:
- NaBH₄: Sodium borohydride
- Blocker: Blocking agent
- NaBH₄ + Blocker: Combination of NaBH₄ and Blocker
Vita

Jeremy Andrew Thompson was born March 6th, 1988 in Richmond, Virginia to James and Sharon Thompson. He received his Bachelor’s of Science in biology and Bachelor’s of Art in French from the College of Humanities and Sciences at Virginia Commonwealth University in December of 2010. He will be attending the Neuroscience Graduate Program at the University of Virginia in the Fall of 2013.